

**Application of Plant Metabolites to overcome Antibiotic Resistance of Methicillin
Resistant *Staphylococcus aureus* (MRSA)**

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ABSTRACT

This study was designed to study the effects of ethyl acetate extracts from *A. wilkesiana* (9EA) and *D. grandiflora* (75EA-L and 75EA-B) and the respective bioactive fractions from these plants on methicillin resistance *Staphylococcus aureus* (MRSA ATCC 43300). A bioassay-guided isolation was used for fractionation of the crude extracts by combinations of liquid chromatography methods. The minimum inhibitory concentration (MIC) of crude extracts and fractions ranged between 12 to 0.75 mg/ml for MRSA and 6 to 0.75 mg/ml for methicillin sensitive *S. aureus* (MSSA ATCC 11630). The MIC values of beta-lactam antibiotics against MRSA strain (i.e. MIC of ampicillin = 50 µg/ml) used in this study were higher compared to MSSA (MIC of ampicillin = 6.25 µg/ml). The crude extracts and selected fractions were evaluated for synergistic activity with ampicillin. The kinetic growth curve experiment illustrated that combination of ampicillin and 9EA or 75EA-L or the fractions derived from these extracts (9EA-FC, 9EA-FD, FC-B, and 75EA-L) suppressed MRSA growth markedly. Results of fractional inhibitory concentration (FIC) index interpretation indicated synergism present in combination treatments of ampicillin and the plant test agents (FIC index < 0.05). Two fractions, FC-B and 75EA-L-F10 were identified to reduce MIC of ampicillin from 50 µg/ml to 1.56 µg/ml and 0.78 µg/ml respectively. These fractions were found to inhibit PBP2a production either alone or in combination with ampicillin in Western blot assay which offered a plausible explanation for restoration of ampicillin's activity in combination treatment. The same fractions were investigated in MRSA biofilm study. Results showed that FC-B or 75EA-L-F10 alone inhibited MRSA biofilm production (~70-80% inhibition). Findings from microtiter attachment assay suggested that these fractions prevent cell-surface attachment (more than 90% inhibition) which is the initial step in biofilm formation. Whereas the PBP2a latex agglutination showed occurrence of low level of PBP2a in MRSA biofilm treated with

FC-B or 75EA-L-F10 implicating possible disruption of cell-cell interactions required for microcolonies development. Ampicillin on the other hand has an inferior activity in preventing cell-surface attachment (37.8% inhibition) although it managed to inhibit MRSA biofilm production by 84.5%. A high performance liquid chromatography (HPLC) and phytochemical analysis showed the studied extracts and fractions are complex mixtures of plant metabolites belonging to the class of tannins, saponins, alkaloids, flavonoids, sterols/steroids, and glycosides. The resistance modifying properties and the anti-biofilm action found in this study are attributed to presence of these phytochemicals. Therefore, we propose that metabolites occurring in *A. wilkesiana* and *D. grandiflora* may be good candidates for development of new treatment for MRSA or as an adjuvant for the current antibiotics.

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2. Mukhrizah Othman, Andrew Spowage, Carolina Santiago, Hwei San Loh, Kuan Hon Lim, Christophe Wiart, Teng Jin Khoo, & Kang Nee Ting. 2011. Inhibition of penicillin binding protein (PBP2a) production in methicillin-resistant *Staphylococcus aureus* (MRSA). MPS-Pharmacy Scientific Conference, 21-23/10/2011, Kuala Lumpur, Malaysia
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LIST OF ABBREVIATIONS

Ac	Acetone
ACN	Acetonitrile
AD	Atopic dermatitis
Alt	Autolysin
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
CAP	Community-acquired pneumonia
CDC	U.S Center for Disease Control and Prevention
CFU	Colony-forming units
CHCl ₃	Chloroform
CLSI	Clinical Laboratory Standards Institute
CTLC	Centrifugal thin layer chromatography
CTRL	Control
DHFR	Dihydrofolate reductase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EA	Ethyl acetate
ECg	Epicatechin gallate
eDNA	Extracellular DNA
EDTA	Ethylenediaminetetraacetic acid
EGCg	Epigallocatechin-gallate
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
Et ₂ O	Diethyl ether

EUCAST	European Committee on Antimicrobial Susceptibility Testing
FA	Formic acid
FDA	U.S Food and Drug Administration
FIC	Fractional Inhibitory Concentration
FnBP	Fibronectin-binding protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H ₂ O	miliQ water
He	n-hexane
HPLC	High performance liquid chromatography
HPV	Human Papilloma Virus
HRP	Horse Radish Peroxide
I	Indifference
iMLS _B	Macrolide-lincosamide-streptogramin B
KH ₂ PO ₄	Potassium dihydrogen phosphate
LC	Liquid chromatography
LDS	Lithium dodecyl sulphate
MDR	Multi-drug resistant
MeOH	Methanol
MHB	Muller Hinton broth
MIC	Minimum Inhibitory Concentration
min	Minute
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
MSSA	Methicillin-sensitive <i>Staphylococcus aureus</i>
MTT	Thiazolyl blue tetrazolium dye
MW	Molecular weight
NaCl	Sodium chloride

NaOH	Sodium hydroxide
NIH	National Institute of Health
OD	Optical density
PBP2a	Penicillin-binding protein
PBS	Physiological buffered saline
PCR	Polymerase chain reaction
PIA	Polysaccharide intercellular adhesin
PNAG	Poly-N-acetylglucosamine
QPT-DFP	Quinupristin-dalfopristin
RMA	Resistance modifying agents
Rpm	Rotation per minute
S	Synergy
SCC <i>mec</i>	Staphylococcal Chromosome Cassette <i>mec</i>
SDS	Sodium dodecyl (lauryl) sulphate-polyacrylamide
SSSI	Skin and skin-structure infections
TMB	3, 3', 5, 5'-tetramethylbenzidine
TMP-SMX	Trimethoprim-sulfamethoxazole
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UV	Ultra-violet
VISA	Vancomycin-intermediate <i>Staphylococcus aureus</i>
VLC	Vacuum liquid chromatography
VRSA	Vancomycin-resistant <i>Staphylococcus aureus</i>
WHO	World Health Organization

LIST OF SYMBOLS

%	Percentage
°C	Celsius
μg	Microgram
μl	Microliter
μm	Micrometer
g	Gram
h	Hour
kDa	Kilodalton
kg	Kilogram
L	Liter
mAU	Milliabsorbance units
mg	Miligram
ml	Milliliter
mM	Micromolar
mm	Milimeter
mol	Mole
nm	Nanometer
V	Volt

CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

One of the major health concerns in the 21st century is infections caused by antibiotic-resistant bacteria. These chronic infections require a longer and complex expansive intervention treatment (Alanis 2005). Infections related to antibiotic resistance were initially observed in hospital settings amongst critically ill and immunocompromised patients. More recently, these infections are reported increasing in the community and in certain instances, in live-stock animals (Alanis 2005; Stefani et al. 2012).

One of the most notorious antibiotic resistance bacterium is methicillin-resistant *Staphylococcus aureus* (MRSA) which is a major pathogen causing nosocomial infection (Michel and Gutmann 1997; Jarvis 2010; Snider and Rivard 2012). MRSA causes skin infections, wound suppuration, pneumonia, and bloodstream infections that eventually lead to sepsis and fatalities. In the Threat Report by the U.S Center for Disease Control and Prevention (CDC), 80,461 severe MRSA infection cases were reported annually with 11,285 deaths per year in the United States (CDC 2013). In Malaysian hospitals, MRSA infection cases reported between the year 2002 and 2007 were showing an increasing trend with annual MRSA prevalence over 40% (Al-Talib et al. 2007; Ghaznavi-Rad et al. 2010). The molecular mechanisms of resistance exhibited by MRSA strains are diversified and complex which poses' great challenges in controlling and preventing the infections. The bacterium was observed 1) to suppress beta-lactamase, an enzyme that degrades beta-lactam drugs, 2) to have an efflux pump system which exports drug like tetracycline out of the cells and 3) to express altered penicillin-binding protein (PBP2a) which has low binding affinity to antibiotics. Besides that, MRSA strains also exhibit various virulence factors that results in chronic infections. The biofilm forming capacity in MRSA is notably a virulent factor that forms barrier to reduce antibiotic penetration and thus increases resistance in the bacterium (Walsh 2000; Rybank and LaPlante 2005; Tenover 2006). Due

to this reason, biofilm-related infection is difficult to treat. In fact, the ability of these bacterial cells to form biofilm is the main reason MRSA remains as number one cause of nosocomial infections (Costerton et al. 1999; Kwon et al. 2008).

Available treatments for MRSA infections are expanding with usage of drugs such as daptomycin, linezolid, and tigecycline (Livermore 2009). However, the level and complexity of resistance mechanism displayed by this pathogen is evolving simultaneously. Evidently, the emergences of *Staphylococcus aureus* strains that are resistance to glycopeptides have been reported following an increased utilization of the antibiotic (Snider and Rivard 2012). Some studies claimed that MRSA infections have reduced in recent years, but the fact MRSA remained as the most common single multi-drug resistance bacterium (Todd et al. 2009) highlights the needs to develop alternative or complementary therapy.

One of the proposed approaches in overcoming bacterial resistance is synergistic therapy which is the use of antimicrobial agents in combination for treatment (Wagner and Ulrich-Merzenich 2009). A classical clinical example for synergistic treatment for bacterial infections is the usage of clavulanic acid, a microbial natural product with the antibiotic amoxicillin in overcoming penicillinase resistance in bacteria. Clavulanic acid functions as beta-lactamase inhibitor, therefore prevents the bacteria from degrading the antibiotic, amoxicillin (Lee et al. 2003; Elander 2003). Besides microbial source, plant metabolites have been suggested as a potential source of resistant modifying agents (RMA) as they produce variety of small molecules (MW < 500) antibiotics (Hemaiswarya et al 2008). At experimental level, plant metabolites were observed to reverse resistance mechanisms displayed by bacteria such as 1) modification of active site of the target, 2) modified enzyme produced by the bacteria or 3) efflux pump (Van Veen et al. 1996; Shiota et al. 2004; Zhao et al. 2001). This supports the hypothesis that plants may serve as a

source of RMA. In addition, plants are known to produce metabolites as flavonols, flavones, terpenoids, and proanthocyanidins that have antibacterial action which is linked to biofilm inhibiting capacity (Koo and Jeon 2009; Kim et al. 2013). Metabolites from these classes have been found to have membrane permeabilizing properties (Abreu et al. 2012) that potentially are able to disrupt the biofilm formation or to enhance permeation of antimicrobial molecules into the cells. In certain cases, combination of plant-derived compound with conventional antibiotic has shown to have synergistic effect in preventing biofilm formation in MRSA (Olson et al. 2011).

The large collection of plants species provides a rich source of antimicrobial compounds (Cowan 1999; Saleem et al. 2010). However, it is important to carefully select a plant source that may be able to yield useful compounds. Selecting a plant source with traditional medicinal use is one important criterion as the medical values these plants are often related to the presence of bioactive metabolites (Cheng et al. 2006; Cos et al. 2006). In this study, two medical plants were selected, *Acalypha wilkesiana* and *Duabanga grandiflora*. *A. wilkesiana* is a medicinal plant which has been widely utilized for treating bacterial and fungal infections in the African regions (Alade and Irobi 1993). In some cases, the plant has been reported to treat malaria and gastrointestinal problems (Akinde and Odeyemi 1987). The plant *D. grandiflora* whereas, is traditionally used for stomach pain and skin diseases especially, eczema (Anderson 1986; Shankar and Devalla 2012).

1.2 LITERATURE REVIEW

1.2.1 Methicillin resistant *Staphylococcus aureus* (MRSA)

Staphylococcus aureus, is a ubiquitous bacterium species commonly found in healthy individuals. The bacterium is known to colonize anterior nares and other skin area in humans. Approximately 50% of adults are *S. aureus* carriers either persistently or intermittently (Wertheim et al. 2005). Although *S. aureus* is commensal in humans, the bacterium is frequently identified to cause wide range of infections involving all organ systems (Archer 1998). In 1880's, Sir Alexander Ogston, postulated *S. aureus* is the major cause of wound suppuration in hospitals (Ogston 1882). This was later observed true, when 82% of death occurred among patients infected by *S. aureus* in Boston City Hospital (Skinner and Keefer 1941). More evidence was found in 1960's at John Hopkins Hospital, after a handful of patients with *S. aureus* bacteremia died even though the infecting organism was tested susceptible to administered antibiotics *in vitro* (Cluff and Reynolds 1965). At this stage, *S. aureus* has started gaining attention as a major cause of nosocomial infections and there was an increase in *S. aureus* infection cases worldwide as a result of antibiotic resistance (Archer 1998).

Initially, a beta-lactam drug, penicillin served as curing drug for *S. aureus* infections. The main structural feature of the beta-lactam antibiotics is the presence of a beta-lactam ring which is responsible for inhibition of bacterial cell wall synthesis by binding to the transpeptidase enzyme. A transpeptidase enzyme functions to catalyze the cross-linking between peptidoglycan chains in bacterial cell wall synthesis. Failure to form this linking cause improperly made cell walls that finally burst due to water flow into the cells (Sabath 1982; Heesemann 1993). However, usage of penicillin was short lived as some bacterial strains beginning to exhibit resistance to penicillin (as early as in 1940's)

by production of a specific enzyme, beta-lactamase or penicillinase that is capable of hydrolyzing peptide bonds in the beta-lactam ring of the antibiotic. Following production of beta-lactamase, *S. aureus* infections were observed to spread rapidly (Lyon and Skurray 1987).

In 1959, a modified penicillin molecule called methicillin was introduced to counteract the deleterious effect of beta-lactamase. Methicillin was designed to bind to penicillin-binding proteins (PBPs) which are a group of enzyme that are necessary for assembly of peptidoglycan chain in latter stage of cell biosynthesis. Binding of methicillin to these PBPs deactivates their function to catalyze the mechanism involved in assembly of peptidoglycan chains. Thus, causing deformities among the bacterial cells which eventually rupture following a weak structure that cannot withhold the osmotic pressure (Stapleton and Taylor 2002; Lambert 2004). Nevertheless, in a very short period, the bacteria developed resistance to methicillin too (Jevons 1961). This time *S. aureus* was seen to express a more complex mechanism in overcoming methicillin's action that is by production of a modified PBP which is PBP2a. PBP2a has a much low binding affinity to methicillin, therefore it evades the antibiotic's action. Due to this resistant factor, *S. aureus* continues to survive despite the presence of methicillin since PBP2a ensures normal synthesis of bacterial cell wall when other PBPs are being blocked by the antibiotic (Katayama et al. 2000). Occurrence of PBP2a in *S. aureus* gave rise to methicillin-resistant *Staphylococcus aureus* (MRSA) which its hallmark is characterized not just by its resistance to methicillin but also to all beta-lactam antibiotics, including synthetic penicillins, cephalosporins and carbapenems (Pantosti and Venditti 2009). This particular resistant factor resulted in sporadic outbreak of nosocomial infections and by the late 1980's MRSA became an endemic pathogen in hospitals (Panlilio et al. 1992).

1.2.2 Molecular epidemiology of methicillin resistance

The major factor contributing to methicillin (and other beta-lactams) resistance in MRSA is the production of PBP2a. PBP2a in MRSA is encoded by the *mecA* gene which was first discovered in 1981 (Hartman and Tomasz 1981). Unlike the beta-lactamase gene, *mecA* is not coded in plasmid but embedded in chromosome, within a genomic island called Staphylococcal Chromosome Cassette *mec* or *SCCmec* (Katayama et al. 2000).

Previous model of *mecA* regulatory mechanism was explained by presence of *mecR1-mecI* genes coding for a sensor-inducer and a repressor respectively (Hiramatsu et al. 1992). In the presence of beta-lactam antibiotics, these regulatory genes are activated due to binding that occurs between the antibiotic and the sensor-inducer, MecR1. This triggers a unique series of proteolytic steps that cleaves the MecI repressor. The cleaved MecI repressor loses its function which is to bind to *mecA* promoter. Thus, enabling expression of the *mecA* gene as long as the antibiotic present (Zhang et al. 2001; Archer et al. 2001). However, several studies have shown that the induction of *mecA* by *mecR1-mecI* system happens exceptionally slow and it is inefficient (Hiramatsu et al. 1992; Kuwahara-Arai 1996). A recent study has revealed that *mecA* locus actually contains a three component system, a newly identified *mecR2* in addition to *mecR1* and *mecI* (see Figure 1.1). The MecR2 functions as an anti-repressor by directly engaging with MecI (repressor) and interrupt its binding to the *mecA* promoter. Hence, enables a complete activation of *mecA* gene for optimal expression of beta-lactam resistance, such as PBP2a production (Arède et al. 2012). Figure 1.1 depicts the proposed model of *mecA* regulatory genes comprised of *mecR1-mecI-mecR2* system.

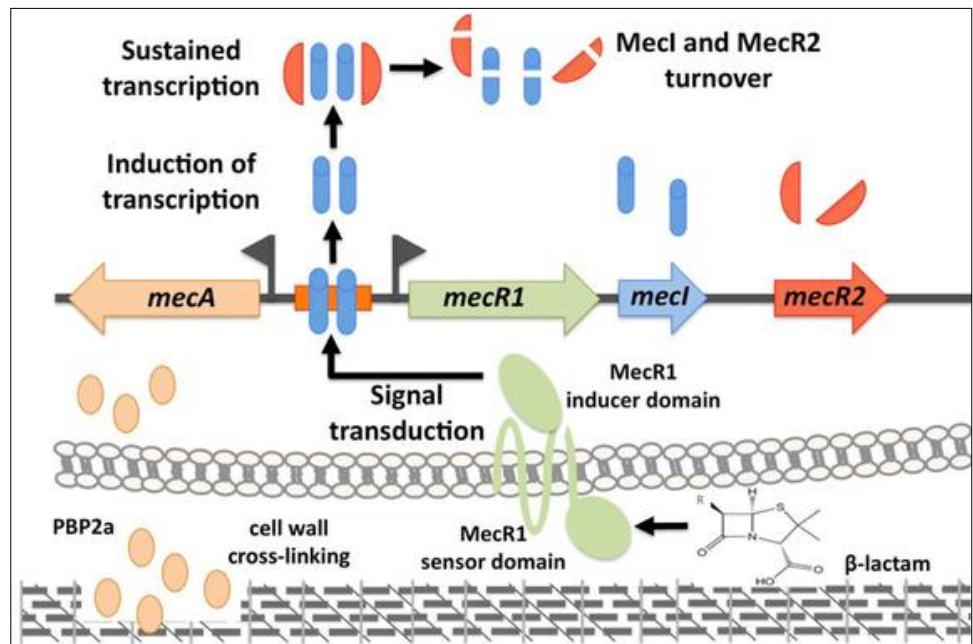


Figure 1.1

Model for the *mecA* induction by Mec1-MecI-MecR2. In the presence of a beta-lactam antibiotic, MecR1 is activated and rapidly induces the expression of *mecA* and *mecR1-mecI-mecR2*. The anti-repressor activity of MecR2 is essential to sustain the *mecA* induction since it promotes the inactivation of MecI by proteolytic cleavage (Arède et al. 2012).

Another regulating system that can control *mecA* gene transcription is *blaI-blaR1* genes which regulate the expression of *blaZ* gene that is responsible for production of beta-lactamase (see Figure 1.2). This phenomena indicates that *blaI-blaR1* induction system controls both PBP2a and beta-lactamase production in MRSA (Hackbarth and Chambers 1993; Sharma et al. 1998). Synthesis of beta-lactamase in *S. aureus* species has been well established previously. Apparently, a beta-lactam antibiotic binds to BlaR1 and triggers the autocatalytic switch of intracellular zinc metalloprotease domain of BlaR1 from an inactive proenzyme to an active protease. The BlaR1 which is now activated cleaves BlaI (the repressor protein) and causing it to become fragments that are no longer capable of its function. With BlaI being fragmented, transcription of *blaZ* and *blaR1-blaI* occurs to commence production of beta-lactamase (Dyke and Gregory 1997; Zhang et al. 2001). Beta-lactamase and PBP2a despite being genetically and chemically distinct, the regulatory proteins of these two (BlaR1-BlaI and MecRI-MecI) was found homologous of each other. Furthermore, the arrangements of gene coding for BlaR1 and BlaI mimic the *mecA* system and the similarity between the operator region of *mecA* and *blaZ* permits BlaI to regulate PBP2a expression (Song et al. 1987; Zhang et al. 2001; Stapleton and Taylor 2002). In fact, the regulatory systems of beta-lactamase and PBP2a were reported to cross-talk (Hackbarth and Chambers 1993), which explains the interrelatedness of these two resistant factors in MRSA. Figure 1.2 shows induction of beta-lactamase production in *S. aureus* species by presence of penicillin (Lowy 2003).

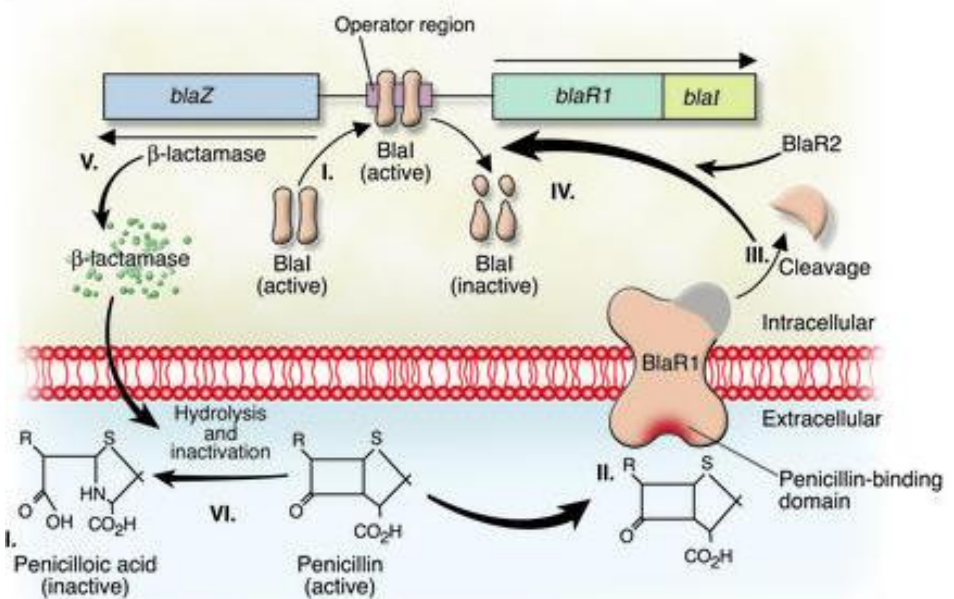


Figure 1.2

Induction of staphylococcal β -lactamase synthesis in the presence of the β -lactam antibiotic penicillin. I. The DNA-binding protein BlaI binds to the operator region, thus repressing RNA transcription from both *blaZ* and *blaR1-blaI*. In the absence of penicillin, β -lactamase is expressed at low levels. II. Binding of penicillin to the transmembrane sensor-transducer BlaR1 stimulates BlaR1 autocatalytic activation. III–IV. Active BlaR1 either directly or indirectly (via a second protein, BlaR2) cleaves BlaI into inactive fragments, allowing transcription of both *blaZ* and *blaR1-blaI* to commence. V–VII. β -Lactamase, the extracellular enzyme encoded by *blaZ* (V), hydrolyzes the β -lactam ring of penicillin (VI), thereby rendering it inactive (VII) (Lowy 2003).

Mechanisms of resistance displayed by MRSA are not limited to production of beta-lactamase and PBP2a. Since MRSA is resistant to beta-lactams, glycopeptides antibiotic, namely vancomycin is widely used to treat MRSA infections. Similar to previous incidents, this was followed by emergence of vancomycin-resistant *S. aureus* (VRSA) in 2002 (CDC 2012). These particular strains are suspected to carry *vanA* gene complex encoding for thicker cell wall, reduced production of PBPs and changes in peptidoglycan biosynthesis. The strains were observed to form lesser cross-linking of peptidoglycan which leaves more protein residues available to bind and trap vancomycin (Hiratmasu et al. 1997; Hanaki et al. 1998). Thus, prevent the drug from reaching its target on cytoplasmic membrane (Sieradzki et al. 1999; Walsh and Howe 2002; Maor et al. 2009). Apart from this, MRSA express resistance to more antibiotics as listed in Table 1.1 (adapted from Lowy 2003). This show extensive adaptation by the bacterium to counteract antibiotic action, suggesting the shelf-life of currently available antibiotics may likely be limited (Lowy 2003).

Table 1.1 Mechanisms of *S. aureus* resistance to antimicrobial agents (Lowy 2003).

Antibiotics	Resistance gene(s)	Gene product(s)	Mechanism(s) of resistance
Beta-lactams	1) <i>blaZ</i>	1) Beta-lactamase	1) Enzymatic hydrolysis of beta-lactam nucleus
	2) <i>mecA</i>	2) PBP2a	2) Reduced affinity for beta-lactams
Glycopeptides	1) Unknown	1) Altered peptidoglycan	1) Trapping of vancomycin in the cell wall
	2) Unknown	2) D-Ala-D-Lac	2) Synthesis of dipeptide with reduced affinity for vancomycin
Quinolones	1) <i>parC</i>	1) ParC (or GrlA) component of topoisomerase IV	1,2) Mutations in the QRDR region, reducing affinity of the enzyme-DNA complex for quinolones
	2) <i>grA</i> or <i>grB</i>	2) GyrA or GyrB components of gyrase	
Aminoglycosides (e.g., gentamycin)	Aminoglycosides-modifying enzymes (e.g., <i>aac</i> , <i>aph</i>)	Acetyltransferase, phosphotransferase	Acetylating and/or phosphorylating enzyme modifying aminoglycosides
Trimethoprim-sulfamethoxazole (TMP-SMZ)	1) Sulfonamide: <i>sulA</i>	1) Dihydropteroate synthase	1) Overproduction of <i>p</i> -aminobenzoic acid by the enzyme
	2) TMP- <i>dfrBrrn</i>	2) Dihydrofolate reductase (DHFR) 23sRNA	2) Reduced affinity for DHFR, mutations in domain V of 23sRNA component of the 50s ribosome interferes with ribosomal binding
Quinupristin-dalfopristin (Q-D)	1) Q: <i>ermA</i> , <i>ermB</i> , <i>ermC</i>	1) Ribosomal methylases	1) Reduce binding to the 23S ribosomal subunit
	2) D: <i>vat</i> , <i>vatB</i>	2) Acetyltransferases	2) Enzymatic modification of dalfopristin

1.2.3 MRSA biofilm as virulent factor

Biofilms are known as surface-adhering bacterial communities encapsulated in extracellular complex comprising DNA, bacterial polysaccharides and proteins by forming a slimy layer (Costerton et al. 1999; Mulcahy et al. 2008). There are several steps required in the development of biofilm. In the initial step, bacterial cells approach to close proximity of a surface till their motility is slowed down. Then they attach to the surface which the process is termed as cell-surface attachment. Following the cell-surface attachment, the bacterium establishes cell-cell interactions to form microcolonies for stability purposes. These microcolonies then, contribute towards development of multilayer structure that eventually leads to a three-dimensional biofilm matrix. With time, when the condition becomes unfavorable some microcolonies may detach itself from the matrix (O'Toole et al. 2000; Watnick and Kotler 2000). In summary biofilm development can be explained in two important steps; 1) primary attachment, the bacteria attach to the surface to be colonized and 2) accumulative phase in which the bacteria form multilayer structure via cell-cell interactions (Mack 1999; Götz 2002; Mack et al. 2004). Figure 1.3 shows a schematic diagram of *S. aureus* biofilm formation (Aslam 2008).

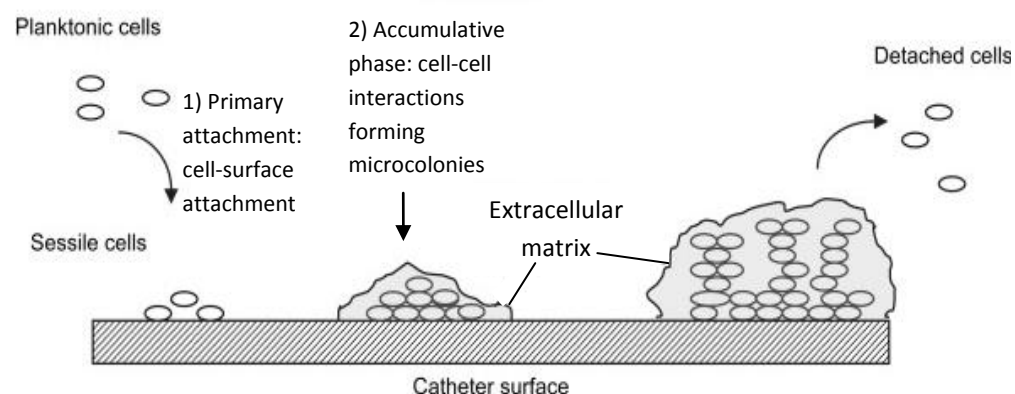


Figure 1.3 An illustration of steps in development of bacterial biofilm formation in *S. aureus* species on a catheter surface. The two vital steps are labeled as 1) and 2) (Image modified from Aslam 2008).

MRSA are often found colonizing implanted prosthetic biomaterials and extensive use of these medical devices for diagnostics and therapeutic purposes contributes to spread of device-related infection involving biofilms in hospitals (Pozzi et al. 2012). A biofilm-related infection is difficult to treat not just because of presence of physical barrier that reduces antibiotic penetration but different growth mode of the biofilm cells that induces expression of antibiotic resistance. The different growth mode in biofilm cells determines the types of antibiotic resistance that is unique and distinct compared to conventional antibiotic resistance mechanism which involves gene mutation or genetic exchange (del Pozo and Patel 2007). In MRSA, this is a main concern, since the bacterium is already resistant to many antibiotics and combination of this with biofilm-associated resistance result in chemotherapeutic failure (Stewart and Costerton 2001; Kwon et al. 2008).

The antibiotic resistance related to bacterial biofilm is caused by multiple factors as summarized in Figure 1.4. Firstly, the presence of biofilm matrix reduces antibiotic penetration. As such, the concentration of antibiotic in the biofilm matrix is low, and

repeated exposure to the low concentration in a long run increases the chances of acquiring resistance against the challenging antibiotic in the bacteria. Also, in other cases exposure of low concentration of antibiotic in *Staphylococcus epidermis* strains were shown to induce *ica* genes that are involved in production of biofilm, therefore low concentration of antibiotic actually encourages a higher biofilm formation (Kwon et al. 2008). On the other hand, in certain bacterial species, antibiotic uptake was observed not to be reduced by presence of biofilm. However, high bacterial density in the microcolonies causes accumulation of waste products and changes the microenvironment such as the pH and oxygen levels which reduce the efficacy of antimicrobial agents (Tack and Sabath 1985; Walter et al. 2003). In *Klebsiella pneumonia*, it was suggested that biofilm traps the antimicrobial agents where they will be inactivated by resistance enzymes such as beta-lactamase (Anderl et al. 2000).

Research has also shown that presence of extracellular DNA (eDNA) in biofilm matrix induces the expression of antibiotic resistance genes. Apparently, the ability of eDNA to bind to cations found in the surrounding gives an environmental cue to the bacteria to induce genes involved in alteration of cell surface component. Eventually, causing modification of bacterial outer membrane that blocks uptake of an antibiotic (Mulcahy et al. 2008). Other factors such as quorum sensing signaling and altered growth rate as in persister cells likewise contribute to biofilm associated antimicrobial resistance. Persister cells are slow or non-growing cells deeply embedded in biofilm (Lewis 2001). Use of antibacterial agents may be able to eradicate majority of the susceptible cells, but the persister cells continue to survive. Upon discontinuation of the antibiotic, these cells give rise to formation of biofilm again (del Pozo and Patel 2007). Combination of these factors ultimately results in high antibiotic resistance in biofilm in which some bacterial cells were up to 1000-fold more resistant to antibiotics compared to planktonic cells (Stewart and Costerton 2001; Mulcahy et al. 2008).

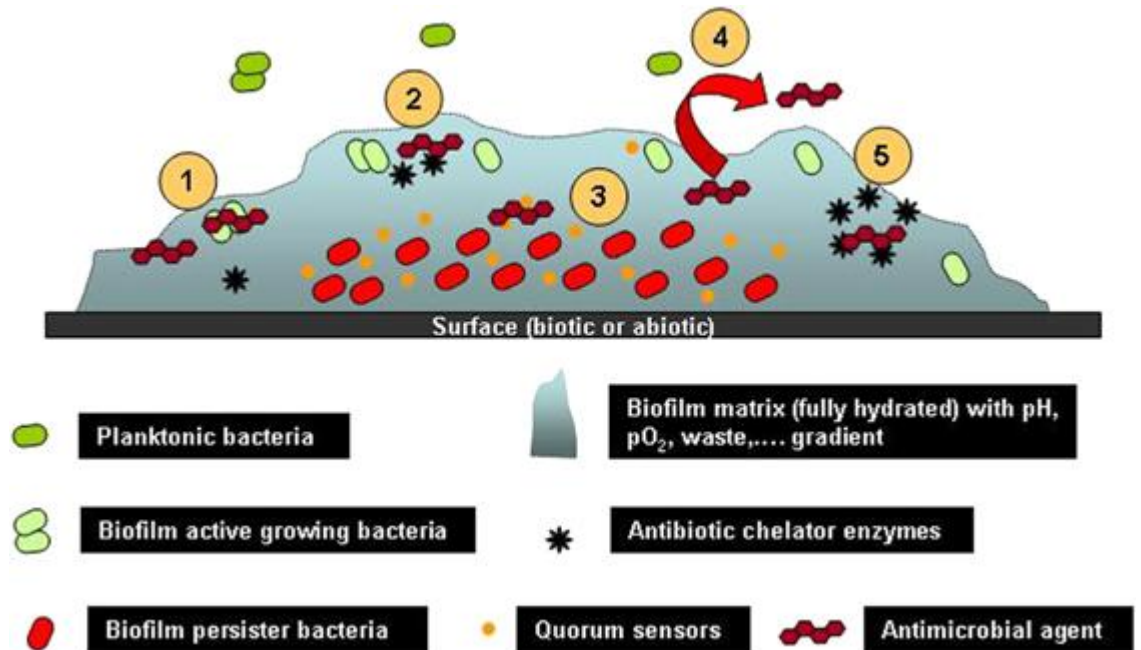


Figure 1.4

Some proposed-biofilm associated resistance mechanisms: (1) Antimicrobial agents may fail to penetrate beyond the surface layers of the biofilm. Outer layers of biofilm cells absorb damage. Antimicrobial agents' action may be impaired in areas of waste accumulation or altered. (2) Antimicrobial agents may be trapped and destroyed by enzymes in the biofilm matrix. (3) Altered growth rate inside the biofilm. Antimicrobial agents may not be active against nongrowing microorganisms (persister cells). (4) Expression of biofilm-specific resistance genes (e.g., efflux pumps). (5) Stress response to hostile environmental conditions (e.g., leading to an over expression of antimicrobial agent-destroying enzymes) (del Pozo and Patel 2007).

Bacterial biofilms are shown to consist of important structural components such as polysaccharides, proteins and DNA (Rice et al. 2007; Boles and Horswill 2008; Otto 2008). Typically, biofilm production in methicillin-sensitive *S. aureus* (MSSA) is dependent on exopolysaccharide intercellular adhesion/polymeric N-acetyl-glucosamine (PIA/PNAG) that are regulated by proteins coded by *icaADBC* genes (Fitzpatrick et al. 2005; Hennig et al. 2007). MRSA strains on the other hand; their biofilm production is mediated by cell wall autolysin (Alt) and fibronectin-binding proteins (FnBPs). In a recent research, high level of PBP2a expression in MRSA strains was found to result in pleiotrophic effect by repressing *icaADBC* dependant polysaccharide type biofilm and promotes protein-mediated biofilm.

The mechanisms responsible for association between MRSA and MSSA biofilm phenotypes and dependency on polysaccharides and proteins, respectively is unknown. Nevertheless, resistance to beta-lactam antibiotics particularly expression of PBP2a seemed to be crucial in determination of the biofilm phenotype (McCarthy et al. 2015). The expression of PBP2a on altered surface of MRSA cells were postulated to facilitate cell-cell interaction (i.e. via altered cell wall architecture) that is essential for multilayer formation in biofilm development (Pozzi et al. 2012). In addition, efficient production of exoprotein (functions as adhesive in biofilm development) and increased FnBPs (a protein that promotes biofilm maturation) in PBP2a-mediated biofilm were implied as advantageous for MRSA in clinical settings to invade host cell or evade the immune system (Saravia-Otten et al. 1997; Vuong et al. 2000; Edwards et al. 2010). This phenomenon not just represents the interrelatedness between methicillin-resistance and biofilm in MRSA strains, but the extraordinary capability of MRSA to switch to protein based biofilm rather than PNAG. This switch enables the bacteria to effectively adapt and colonize implanted medical device for treatments.

1.2.4 Therapeutic options for MRSA

At present, limited options are available for patients infected with MRSA due to increase in antimicrobial resistance. Glycopeptides, namely vancomycin has been a choice of drug for MRSA infection since 1960. However as described in section 1.2.2, the emergence of VRSA showed that vancomycin is no longer the “gold standard” to treat MRSA infections (Snider and Rivard 2012). Recent studies suggested that vancomycin has slow bactericidal activity, may possibly promote development of drug resistance (Deresinski 2007; Mohr and Murray 2007; Liu et al. 2011). There is also increasing reports of treatment failures with usage of vancomycin showing that MRSA is progressively becoming resistant to the drug (Deresinski 2007; Rivera et al. 2011).

Linezolid is another antibiotic approved for of nosocomial pneumonia caused by MRSA. When administered orally, the drug has 100% bioavailability and additionally has excellent penetration into epithelial lining fluid making it efficient for treatment of pneumonia. Therefore, linezolid has better clinical cure rates compared to vancomycin for MRSA infected pneumonia (Wunderink et al. 2003; Kollef et al. 2004). There is another study stating that the drug is unable to inhibit toxin production in the bacterium which then contributes to an increased severity in the infections (Takahashi et al. 2010).

Daptomycin is cyclic lipopeptide drug that acts by depolarizing cell membrane which causes inhibition of protein synthesis in bacteria. It is recognized for treatment of chronic MRSA infections excluding pneumonia due to adverse effect in respiratory system (Liu et al. 2011; Rivera et al. 2011). The bactericidal activity is concentration-dependant, thus some support implement of a higher dosage (Benvenuto et al. 2006; Figueroa et al. 2009). However, high doses of daptomycin may induce myopathy (Liu et al. 2011). Hence,

the study of optimal dosing of daptomycin needs further evaluation to limit the adverse effects of the antibiotic (Snider and Rivard 2012).

Telavancin is a derivative of vancomycin, thus the mode of action is similar to vancomycin which is by inhibition of cell wall synthesis. In addition to that, the drug depolarizes bacterial membrane leading to disruption of barrier function. Telavancin is currently used to treat MRSA and vancomycin-intermediate *S. aureus* (VISA) infections (Astellas Pharma 2012). Certain reports suggested that the drug potentially has part in biofilm-related infections since patients with infective endocarditis (a disease that is difficult to treat due to biofilm production) were successfully treated with telavancin although the drug is not indicated for treatment (Howden et al. 2010; Marcos et al. 2010; Nace and Lorber 2010). Telavancin contraindicated in pregnant women following findings in animal studies that revealed its teratogenic effect (FDA 2011).

Tigecycline is indicated for treatment of skin and skin-structure infections (SSSI), intra-abdominal infections and community-acquired pneumonia. Mode of its antibacterial effect is inhibition of protein synthesis. In order to avoid major bacterial resistance, tigecycline structure was designed with a modified side chain (Lexi-Comp 2011; Snider and Rivard 2012). This antibiotic is particularly advocated effective for treatment of serious MRSA infections by researchers that conducted clinical study (Florescu et al. 2008). Nevertheless, analysis of 7400 patients in a clinical trials indicated a higher risk of all-cause mortality with the utilization of tigecycline to treat serious infections which followed by warning from FDA to use an alternative agent (FDA 2012).

Ceftaroline is an antibiotic belonging to class of cephalosporin and has wide-spectrum activity against MRSA, VISA and VRSA (Snider and Rivard 2012). In treatment of SSSI, the drug was found to have relatively good activity similar to vancomycin (Corey et

al. 2010). Most importantly, the drug has the same safely profile as other cephalosporins but with an added advantage of remaining active against resistant pathogen like MRSA and VISA (Biek et al. 2010; Steed et al. 2010).

Quinupristin-dalfopristin (QPT-DFP), a combined streptogramin drug targeted to interrupt protein synthesis in bacteria (Drew et al. 2010). Often, the combined drugs administered to treat SSSI caused by MSSA (Lexi-Comp 2011). In certain instances, when MRSA infections are incurable with other antibiotics, QPT-DFP is used. However due to severe myalgias as side-effect, the use of QPT-DFP is very limited (Olsen et al. 2001).

Trimethoprim-sulfamethoxazole (TMP-SMX) is a combination of two antibiotics that is able to suppress folic acid synthesis in the bacteria. Each of these antibiotics separately inhibits enzymes involved in folic acid synthesis that contributes to additive effect in preventing bacterial growth (Grim et al. 2005). Despite not being approved by FDA for treatment of MRSA infections, the drug is widely administered for treatment of noninvasive community-acquired MRSA infection (Liu et al. 2011). Findings in clinical studies that compared treatment of vancomycin and TMP-SMX, concluded that, therapeutic efficacy of TMP-SMX is lower than vancomycin.

Tetracyclines (including doxycycline and minocycline) are antibiotics which function as bacteriostatic agents by inhibiting protein synthesis in bacteria (Liu et al. 2011). Originally this class of antibiotics used to treat SSSI associated with *S. aureus*. Emergence of MRSA strains carrying *tetK* gene, exhibited efflux pump system that flushes the drug out the cells, making the tetracyclines drugs ineffective (Ruhe et al. 2005; Ruhe et al. 2007). However, minocycline was observed to have a higher capabilities compared to other tetracyclines in overcoming resistance mechanism displayed by MRSA due to its longer half-life, better absorption and enhanced tissue-penetration (Bishburg and

Bishburg 2009; Liu et al. 2011). Besides, minocycline was found to be highly active against MRSA isolates in biofilm compared to vancomycin, daptomycin, linezolid and tigecycline (Bishburg and Bishburg 2009). Despite the encouraging activity of minocycline against MRSA, the side effects of this drug is serious including worsening azotemia in patients with renal disorders, pancreatitis, autoimmune hepatitis and drug-induced lupus-like-syndrome (Bishburg and Bishburg 2009). Based on the emergence of antibiotic resistance trends, tetracycline-resistance in MRSA strains may soon affect minocycline.

Clindamycin indicated for treatment of *S. aureus* infections and frequently used to cure SSSI. The drug's mechanism of action is due to interference of the transpeptidation process that results in protein synthesis inhibition. Clindamycin however, is not approved by FDA for treatment related to MRSA. Even so, the antibiotic was shown to have great success in curing invasive community-acquired MRSA infections in some cases. Clindamycin is bacteriostatic agent hence, not suitable for all types of MRSA infections (Lexi-Comp 2011; Liu et al. 2011). MRSA strains that resistant to erythromycin but susceptible to clindamycin are prone to exhibit macrolide-lincosamide-streptogramin B (iMLS_B). The iMLS_B strains can be induced by erythromycin to produce methylase that give rise to clindamycin resistance (Siberry et al. 2003).

Based on the discussion above, it is evident that MRSA has developed resistance towards some of the older drugs such as vancomycin and tetracyclines. The newer drugs are potentially effective for MRSA infections but the evidence from the past shows a disturbing fact; an increase in usage of antimicrobial agent results in a display of more complex resistance mechanism in the pathogens (Krause 1992; Tenover 2006). Large number of side effects manifests the toxicity of these antibiotics. Table 1.2 is showing FDA-approved drugs for MRSA infections. From the table, we can deduce that a small

number of antibiotics are available to treat nosocomial pneumonia, bacteremia, infective endocarditis, and complicated intra-abdomen infections. This suggests that the development of new antibiotic agent or adjuvant for the current antibiotics is necessary to successfully treat MRSA infections.

Table 1.2 FDA-Approved Indications for MRSA Infections (Snider and Rivard 2012).

Drug/ Infections	Complicated SSSI	CAP	Nosocomial Pneumonia	Bacteremia	Infective Endocarditis	Complicated Intra- abdominal Infections
Vancomycin	X	X	X	X	X	
Linezolid	X	X	X			
Daptomycin	X			X	X	
Tigecycline	X	X				X
Telavancin	X					
Ceftaroline	X	X				

SSSI: skin and skin-structure infections

CAP: community-acquired pneumonia,

Quinupristin-dalfopristin, clindamycin, doxycycline, minocycline and trimethoprim-sulfamethoxazole do not have FDA- approved indications for the treatment of MRSA infections.

1.2.5 Concept of synergism for treatment of antibiotic resistance

The antibiotic discovery process has failed to keep up with the evolution pace of antimicrobial resistance in bacteria. Hence, it is necessary to find new strategies to develop antibiotic in order to effectively control the spread of antimicrobial resistant and to cure for serious infections (Abreu et al. 2012). Synergy interaction between combinations of two antimicrobial agents is identified as a potential way to solve resistance problems. This is because mode of action of a combination of two agents differs extensively compared to the action of the agents when used separately (Hemaiswarya et al. 2008). In combination therapy, two agents act synergistically by 1) having multi-targets, which means the components in the combination affect several targets and hence results in agonistic effects or potentiated pharmacological effects, 2) pharmacokinetic effects due to enhanced solubility and resorption rate as a result from presence of one of the agent that is able to increase bioavailability of the other agent hence promotes a better activity, 3) interference with resistance mechanism of the bacteria by one agent amplifies the activity of the other in the combination, and 4) elimination of toxin factors by one of the agents that increases inhibitory activity of the other (Wagner and Ulrich Merzenich 2009).

The use of drug in combination is rather common in clinical settings to maintain clinical efficacy and to overcome resistant problems since combination of certain drugs results in higher inhibitory effects compared to individual drug's potency (Torella et al. 2010). For example, treatment for infections caused by multi-drug resistant *Pseudomonas aeruginosa* often consists of an anti-pseudomonal beta-lactam and an aminoglycoside or a fluoroquinolone (Pier et al. 2005). In *S. aureus* related infections, combination of cell-wall active antibiotic such as penicillins and glycopeptides with aminoglycosides are widely use for treatments (Goldstein et al. 2003). While it is shown clinically that combination of

drugs increase the effectiveness of treatments in selected cases, it is important to understand the impact of synergy drug treatment on evolution of antimicrobial resistance. Results from a study using a mathematical simulation of an *in vivo* infection model revealed that drug synergism may worsen antimicrobial resistance (Torella et al. 2010). This finding echoes an earlier claim that overuse of antibiotics is one of the major factors causing emergence of resistance amongst bacteria (World Health Organization [WHO] 2011).

Most of the available antibiotics today are of bacterial or fungal origin and with growing concern over resistance issues and toxicity; the interest in finding antibacterial agents is shifted from microbial source to plant (Cowan 1999). In addition many studies suggest that plants have effective innate defense system that is able to overcome resistance expressed by bacteria due to the synergistic interaction between the produced metabolites (Lewis and Ausubel 2006; Sibandah and Okoh 2007; Hemaiswarya et al. 2008). This proposal was based on the ability of plants to successfully overcome infective diseases despite producing weak antibacterial agents. Hence, a strategy of combining plant products with antibiotics is actively studied to identify potential RMAs (Abreu et al. 2012).

Many plant products were observed to work synergistically with antibiotics in inhibiting growth of *S. aureus* strains, MSSA and MRSA, and some examples of the plant metabolites and their activities are listed in Table 1.3. It is evident that plant products have enormous potential to be developed as antibacterial agents or as adjuvants for current antibiotics since they are able to interfere with mechanisms of resistance such as inactivation of multi-drug resistant (MDR) efflux pump, inhibition of PBP2a, and inhibition of penicillinase/beta-lactamase (Table 1.3).

Table 1.3 Selected synergistic activities between plant products and antibiotics adapted from Hemaiswarya et al. (2008) showing inhibition of *S. aureus* strains, MSSA or MRSA.

Plant products	Antibiotics	Bacteria	Mechanism of action	References
Epigallocatechin-gallate (EGCg)	Ampicillin/ sulbactam	MSSA, and beta-lactamase producing <i>S. aureus</i> strains	Inhibits beta-lactamase	Hu et al. 2001
EGCg	Penicillin	Penicillinase producing <i>S. aureus</i> strains	Inhibits penicillinase	Zhao et al. 2001
EGCg	Beta-lactam antibiotics	MRSA, MSSA	EGCg directly binds peptidoglycan and inhibits cell wall synthesis	Yoshida et al. 1990
EGCg	Tetracycline	<i>S. aureus</i> with TetK gene, multi-drug resistance (MDR) pump	Block efflux pump	Roccaro et al. 2004
Tea catechin	Oxacillin	MRSA	-	Takahashi et al. 1995
Totatrol	Methicillin	MSSA, MRSA	Inhibition of PBP2a production or activity	Pao et al. 1998
Isoflavone Bidwillon B	Mupirocin	MRSA	Affects incorporation of thymidine, uridine, glucose, and isoleucine	Sato et al. 2004
α -Mangostin	Vancomycin	MRSA	-	Sakagami et al. 2005
Corilagin	Beta-lactam antibiotics (oxacillin, cefmetazole)	MRSA	Inhibition of PBP2a production or activity	Shimizu et al. 2001
Baicalin	Beta-lactam antibiotics	MRSA	Inhibits beta-lactamase	Liu et al. 2000
Tellimagrandin I	Beta-lactam antibiotics	MRSA	-	Shiota et al. 2000
Rugosin	Beta-lactam antibiotics	MRSA	-	Shiota et al. 2000

Plant products	Antibiotics	Bacteria	Mechanism of action	References
Diterpernes from <i>Lycopus europseus</i>	Tetracycline	<i>S. aureus</i> with <i>TetK</i> gene, (MDR) pump	Blocks MDR pumps	Gibbons et al. 2003
Penta-substituted pyridine from <i>Jatropha elliptica</i>	Erythromycin	<i>S. aureus</i> with <i>TetK</i> gene	Blocks MDR pumps	Marquez et al. 2005
Pomegranate extract	Ciprofloxacin, chloramphenicol, gentamycin, ampicillin, tetracycline, and oxacillin	MRSA	Blocks Nor (A) pump (an efflux pump)	Braga et al. 2005
Myricetin	Amoxicillin/clavulanate, ampicillin/sulbactam and ceftiofur	MSSA	-	Lin et al. 2005
Isopimaric acid from <i>Pinus nigra</i>	Reserpine	MRSA	Block Nor (A) pump	Simonetti et al. 2004
Erybraedin A or eryzarin C	Vancomycin	MRSA	-	Shiota et al. 2004
Sophoraflavanone	Vancomycin hydrochloride, fosfomicin, methicillin, ceftazidime, gentamycin, minocycline, and levofloxacin	MRSA	-	Sakagami et al. 1998

Synergism of plant metabolites with antibiotics in disrupting bacterial biofilm is not widely studied. However, several studies have demonstrated that plant metabolites act synergistically with antibiotic to suppress biofilm production. For instance, a novel pentadecenyl tetrazole of plant origin was shown to exhibit synergism with gentamycin against both planktonic and biofilm cells of *S. aureus* strain (Olson et al. 2011). In another study essential oils from the plants *Cinnamomum zeylanicum*, *Melaleuca alternifolia* and *Cymbopogon martini* acted synergistically with ciprofloxacin by significantly disrupting biofilm formation in *P. aeruginosa* (Coelho and Pereira 2013). While in Italy, plant extracts, especially with ethnobotanical properties are used to treat SSSI linked to *S. aureus* with growing concern over antibiotic resistance. Some of these plants extracts when tested *in vitro* demonstrated strong inhibition on bacterial biofilm production (Quave et al. 2009). Combining this information, it is obvious that plant may harbor metabolites with anti-biofilm properties.

In a recent study, anti-biofilm activity of plant metabolites namely 7-hydroxycoumarin, indole-3-carbinol, salicylic acid, and saponin against *E. coli* and *S. aureus* biofilms were suggested due their interference with motility and quorum-sensing activity in the bacterial cells. Besides, one of the metabolites, indole-3-carbinol when combined with tetracycline, erythromycin and ciprofloxacin produced a synergistic effect in suppressing growth of *S. aureus* strains which further implied that the interference with cell motility and quorum-sensing activity prospectively enhanced the antibacterial effects. Therefore, promotes restoration of the drugs' antimicrobial potency (Monte et al. 2014). Several plant products were observed to prevent cell-surface attachment as well, which is an essential step in biofilm development. Although, cationic peptides due to their surface charge and hydrophobicity, frequently found to exhibit such effects on biofilm formation (Overhage et al. 2008; Mataraci and Dosler 2012), the compound 1,2,3,4,6-penta-o-galloyl- β -d-glucopyranose, which is an active plant ingredient used in traditional Chinese

medicine, was found to inhibit *S. aureus* biofilm formation in the same manner (Lin et al. 2011). This shows that plant metabolites may have similar actions as cationic peptides in altering the cell-surface attachment adversely.

In summary, research from the past has shown that plant products either alone or in combination with conventional antibiotics are capable on inhibiting bacterial growth. The inhibition of bacterial growth was observed not only on planktonic cells but also biofilm protected cells, which makes plant metabolites as a valuable source of new antibacterial agents since biofilm production contributes to an increased antimicrobial resistance. Mode of action studies suggested in many cases, plant products or molecules specifically interact with the bacterial resistance mechanism, thus, enabling the potentiating of the antibiotics bactericidal effects when used in combinations. For the anti-biofilm action, these metabolites were suggested to disrupt biofilm through suppression of cell motility, quorum-sensing activity and cell-surface attachment.

1.2.6 Selected plant species

The era of drug discovery from plants was initiated after the finding of morphine from *Papaver somniferum* which was the first pharmacologically active pure compound from plants (Hamilton & Baskett 2000). Since then, compounds from plants have gained popularity in the medical field for their application as antimicrobial, anticancer, antidiabetic and immunosuppressant agent (Newman et al. 2003). Among the important classes of biological compounds from plants include acetylenes, coumarins, flavonoids, lignans, phenolics (other than flavonoids and lignans), polypeptides, alkaloids, steroidal saponins, terpenoids and xanthenes (Cowan 1999; Saleem et al. 2010). In this study two medicinal plants were selected; *A. wilkesiana* and *D. grandiflora*.

1.2.6.1 *Acalypha wilkesiana*

A. wilkesiana belongs to the genus *Acalypha* with comprises approximately 570 species (Riley 1963) and it is classified in the Euphorbiaceae family. The plant grows all around the world particularly in tropics of Africa, America and Asia (Madziga et al. 2010). It is a fast growing medium sized shrub. The leaves are 4-8 inches long in heart shape and their color varies in combination of green, purple, red, bronze, yellow, orange, pink, and white depending on the cultivar (Gilman 1999). For this reason, *A. wilkesiana* is normally planted as ornamental plants. Figure 1.5 is showing a photograph of the plant *A. wilkesiana*.



Figure 1.5 A photograph of the plant, *A. wilkesiana*.

In Nigeria and other African countries, this plant has been widely used for its antibacterial and antifungal properties (Akinde & Odeyemi 1987; Alade & Irobi 1993). Traditionally, the juice from this plant has been used to cure ailments such as skin diseases mostly on children (Oliver 1959). The preparation includes boiling the leaves for bathing purposes and a small volume is to be drank (Alade & Irobi 1993). Besides, *A. wilkesiana* is also used to treat other diseases such as malaria and gastrointestinal problems (Akinde & Odeyemi 1987). Another species of Acalypha, *A. hispidia* is used to cure ulcer, abscesses and leprosy (Schindeler 1939). The traditional uses of this plant suggest it possesses antimicrobial properties.

Accordingly, preceding studies have reported antimicrobial activities found in extracts of this plant. The extracts have wide spectrum activity whereby it is able to inhibit growth of both Gram positive and Gram negative bacteria as well as fungi (Oladunmoye 2010). Among the clinical strains that were reported to be inhibited by *A. wilkesiana* extract are *E. coli*, *Salmonella typhi*, *Streptococcus pyogenes*, *Streptococcus pneumonia*, MRSA, *S. aureus*, *Candida albicans*, *Aspergillus fumigatus*, and *A. flavus* (Ezekial et al. 2009). Likewise, in our earlier study, the plant exhibited anti-staphylococcal activities together with wide-spectrum antibacterial activity against other bacteria such as

E. coli and *Citrobacter freundii* (Othman et al. 2011a; Othman et al. 2011b). Whereas, studies by other group in our laboratory demonstrated that extracts from *A. wilkesiana* acted synergistically with ampicillin in inhibiting *S. aureus* growth (Din et al. 2013a) and anti-cancer drug to stop proliferation of cancer cells (Lim et al. 2011; Lim et al. 2013).

Studies on antibacterial effects of *A. wilkesiana* extracts reported presence major classes of phytochemicals such as tannins, steroids, flavonoids, cardiac glycosides, saponins, alkaloids, and antraquinone that were thought to be responsible for the antimicrobial properties of this plant (Oladunmoye 2006; Gotep et al. 2010). Other constituents that were present in the extracts of this plant include carbohydrates, phlobatannins, and minute quantity of terpenes (Madziga et al. 2010). Tannins from *A. wilkesiana* especially gallic acid, corilagin, ellagitannins and geraniin are frequently associated with antimicrobial activities since these compounds are able to cause bacterial cell lysis (Adesina et al. 2000; Din et al. 2013a; Din et al. 2013b).

1.2.6.2 *Duabanga grandiflora*

D. grandiflora (Roxb. Ex DC) Walp, belongs to the Lythraceae family. It is indigenous to Eastern Himalayas and widely distributed in tropical African and Southeast Asia (Graham et al. 2005; Auamcharoen et al. 2009). The tree grows between 40-80 feet high, as one undivided trunk or sometimes forking from the base. The branches are sparing with large spreading leaves in deep green on the surface on almost white beneath. *D. grandiflora* flowers blossoms in April, and has an odor similar to asafoetida initially before the petal drops. The tree also produces fruit with its size almost like a small apple (Hooker et al. 1855). Figure 1.6 is showing a close up photograph of the leaves and fruit of *D. grandiflora* tree.



Figure 1.6 A photograph of the plant, *D. grandiflora*

Traditionally, people from hill tribes in Northern Thailand utilize poultices from its leaves to treat stomach pain (Anderson, 1986). In India, the bark paste of this plant is used to cure skin diseases, mainly eczema (Shankar and Devalla, 2012). *D. grandiflora* extracts are also shown to have skin whitening, anti-aging, anti-inflammation and anti-cancer properties (Bhakuni et al. 1971; Tsukiyama et al. 2010; Kawetripob et al. 2012). The plant however is not widely studied for antimicrobial properties. In fact, our previous

research was the only published work on antibacterial activity of *D. grandiflora* extracts (Othman et al. 2011a; Othman et al. 2011b).

Major phytochemicals such as alkaloids, tannins, flavonoids, saponins, and steroids were detected in *D. grandiflora* extracts that demonstrated antibacterial activities (Othman et al. 2011a). Among the compounds isolated from this plant (*D. sonneratioides* Buch.-Ham-subsequently classified in synonym with *D. grandiflora*) are hentriacontanone, lignoceryl ferulate, acacetin, betulinic acid, sitosterol- β -D-glucoside, ellagic acid, tetramethylellagic acid, epioleonic acid, and genkwanin which are associated with anti-cancer properties (Bhakuni et al. 1971; Sharma et al. 1972a; Sharma et al. 1974). Recently, 5-formylfurfuryl esters, latifoninal, pentacyclic triterpenes, benzofuran derivative, ellagic acid derivatives along with several other known compounds were isolated from *D. grandiflora*. These metabolites likewise exhibited moderate to weak activity anti-cancer activities (Kaweetripob et al. 2012).

1.3 RESEARCH RATIONALE

The fundamental idea of this research is application of secondary metabolites from *A. wilkesiana* and *D. grandiflora* in reversing ampicillin resistance in MRSA. The two targeted virulent factors that we are interested includes: 1) PBP2a that confers resistance to all beta-lactam antibiotics including ampicillin and 2) biofilm production which complicates treatment of MRSA infections by a reduced antibiotic uptake. A strategy to eliminate resistant problems is by adopting synergism approach which is using combinations of antimicrobial agents. The advantages of synergism approach have been extensively described in reviews by Hemaiswarya et al. (2008), Wagner and Ulrich-Merzenich (2009) and Abreu et al. (2012). Briefly, the concept of synergisms can be achieved by four mechanisms 1) multi-target effects that cooperate in agonistic way, 2) pharmacokinetic effects which increase solubility, resorption rate and improve bioavailability, 3) adverse interaction with resistance mechanism, and 4) elimination of toxic factors (Wagner and Ulrich-Merzenich 2009).

Plant metabolites are frequently found to display different mechanisms in contrast to conventional antibiotic in acting against microbial pathogens which can be advantageous in combating antibiotic resistance in some bacteria (Abreu et al. 2012). This has initiated the idea if the synergism between ampicillin and extracts or bioactive fractions from the studied plants is affecting resistant factors in MRSA adversely. The major resistant factor in MRSA is PBP2a production. Inhibition of PBP2a is proposed to increase MRSA susceptibility to ampicillin and restore the antibiotic's efficacy. Secondly, disruption of biofilm formation in sessile bacterial cells is thought to expose the cells to the antibiotic and therefore reinstate the effectiveness of the antibiotic.

The selected plant *A. wilkesiana* has been traditionally used to cure bacterial infections and evidently laboratory studies have shown that extracts of this plant also

demonstrated wide-spectrum antimicrobial activity thus, affirming presence of pharmacologically important metabolites (Ezekial et al. 2009; Oladunmoye 2010). Subsequent studies revealed the finding of compounds such as corilagin, geraniin, ellagitannins and many others that particularly inhibited growth of *S. aureus* and MRSA (Adesina et al. 2000; Din et al. 2013a; Din et al. 2013b). Therefore, this plant is a potentially good candidate. The plant *D. grandiflora* was the second choice of plant, is widely used in India to treat skin diseases associated with eczema or atopic dermatitis (AD) (Shankar and Devalla 2012). Advancement in dermatological research showed *S. aureus* causes skin lesion in AD patients and skin samples from these patients were found to contain *S. aureus* delta toxin (Rudikoff and Lebwohl 1998; Nakamura et al. 2013). These findings indicate that ethnobotanical use of *D. grandiflora* in treating eczema may actually have some evidence to its ability to heal bacterial infections namely *S. aureus*. Hence, the study of combined effects of ampicillin with extracts or fractions from the named plants on MRSA may reveal potential chemotherapeutic value of these plant secondary metabolites in restoring efficacy of the antibiotic.

Earlier studies in our laboratory, we have identified ethyl acetate extracts of these plants as the most potent against MRSA (Othman et al 2011a; Othman et al. 2011b). Following that, experiments in this study focused on exploiting the ethyl acetate extracts.

1.4 RESEARCH APPROACHES

Our research was focused on finding secondary metabolites from *A. wilkesiana* and *D. grandiflora*. The general approaches in this research are divided into two areas; a) the biological studies and b) the application chemistry methods. In the biological approaches, experiments were conducted to 1) evaluate the anti-MRSA activity of the plant extracts and fractions through determination of minimum inhibitory concentration (MIC) value, 2) investigate the synergistic effects of combination of the plant extracts or fractions with ampicillin on MRSA growth and 3) study on the effects of the combination treatments on a resistant factors in MRSA which are i) PBP2a production and ii) biofilm formation. The chemistry approach included 1) extraction of the plant materials, 2) separation of the crude extracts into major fractions and semi-pure fractions by liquid chromatography (LC) methods, 3) determination of presence of major classes of compounds in the crude extracts and bioactive fractions, and 4) application of high performance liquid chromatography (HPLC) to study the complexity of the plant samples.

A bioassay-guided approach was used in this study in which we incorporated biological assays after the extract or fractions were separated via LC methods to include fractions that retained the anti-MRSA activity. These fractions were termed as bioactive fractions. The synergistic studies revealed useful combination of different bioactive fraction with ampicillin via kinetic growth curve experiments and fractional inhibitory concentration (FIC) index interpretations. The growth curve experiment was designed to monitor MRSA growth for a period of 24 hours. Whereas the FIC index interpretations yield a quantitative measure of the interaction between the extracts or fractions and ampicillin to identify any synergism. Furthermore, the FIC index interpretation enables the determination of a different MIC of ampicillin in the combination. The new MIC indicates if the effectiveness of ampicillin has been restored in presence of plant extract or

bioactive fraction. Following these experiments, combinations with the lowest ampicillin concentration, selected and investigated for their mechanism of action.

The mode of action studies were carried out to look at two virulent factors: 1) the expression of PBP2a and 2) disruption of biofilm production. In the Western blot experiment, the combination treatments were studied if they are able to suppress PBP2a expression in MRSA, which is the major cause of beta-lactam resistance in MRSA strains. Whereas, the biofilm forming capacity of MRSA in presence of the combination treatments was investigated by employing assays which enabled quantification of biofilm production via crystal violet staining method. Besides quantifying biofilm production, MRSA cell-surface attachment in presence of the combination treatments was studied likewise. The level of PBP2a in biofilm matrix in the treated cultures was evaluated semi-quantitatively via PBP2a latex agglutination assay. The cell-surface attachment and PBP2a are two factors suggested to contribute towards MRSA biofilm production. These experiments were conducted to partially elucidate the possible mechanism of actions of some the bioactive fractions we have isolated.

1.5 SIGNIFICANCE OF STUDY

The findings from this study are expected to be important in the following areas;

1. Advances in *in vitro* studies of application of plant metabolites as synergist of ampicillin in overcoming resistant problems in MRSA particularly PBP2a and biofilm production. This will enable reuse of conventional antibiotics which are well-established and often cheaper with known safety profile.
2. Evidence-based study of use of medicinal plants which may encourage search for more plant-derived drugs. These drugs often serve as prototypes for chemist to design more effective antibiotics.
3. Potential discovery of active molecules or extracts that can be used to treat topical wound infection with MRSA or coatings for medical implants to prevent biofilm-related infections. In 2006, U.S Food and Drug Administration (FDA) has approved green tea extract as a prescription drug for the topical (external) treatment of genital warts caused by the human papilloma virus (HPV). This drug contains active ingredient known as Polyphenon®. Thus, suggesting plant extracts can be utilized as a potential preparation for infections.

1.6 AIMS AND OBJECTIVES

The studies conducted in this research was aimed to investigate application of plant metabolites from *A. wilkesiana* and *D. grandiflora* in overcoming resistance in MRSA namely through inhibition of PBP2a and biofilm production. The objectives are as the following;

1. To evaluate anti-MRSA activities of the extracts and fractions obtained from the plants via determination of MIC.
2. To determine the synergistic effects of combination treatments consisting ampicillin and selected extracts or fractions on MRSA growth.
3. To investigate the effects of the selected combination treatments on PBP2a and biofilm production in MRSA.
4. To identify major classes of compounds in extracts and fractions from the named plants by phytochemical analysis.
5. To determine fractions that has potent antimicrobial and at the same time is able to retain the activity throughout the studies via bioassay-guided fractionation.

CHAPTER 2

BIOASSAY GUIDED FRACTIONATION AND DETECTION OF PHYTOCHEMICALS

2.1 BACKGROUND

The current situation of public health is continuously being threatened by the emergence of diseases caused by antibiotic resistant bacteria. Resistant infections which are difficult to treat often lead to increased economic burden and fatalities especially in underdeveloped countries (Okeke et al. 2005). Attempts to discover new therapeutics to combat this problem are often associated with the studies of plant extracts as well as pure compounds isolated from the extracts of medicinal plants. A recent trend has shown that global population that is attracted to use medicinal plants or natural remedies to treat various infectious diseases is on the rise (Cos et al. 2006; Tiwari 2008).

Literature reviews showed that the plants *A. wilkesiana* and *D. grandiflora* were traditionally used to treat bacteria associated infections (Alade and Irobi 1993; Shankar and Devalla 2012). Following that these plants were selected for this study in which a bioassay guided approach was employed in order to identify bioactive components. Bioassay guided isolation is a basic yet useful method to search for antimicrobial natural products. It is a process of characterizing substances with biological activities by using chromatography methods for fractionation/separation of extracts and bioassays to identify bioactive fractions/compounds. This method tends to yield valuable information on biological effects, although the bioactivities are not always assignable to a specific chemical compound (Weller 2012) and serves as a basis to discriminate the non-active portions of a mixture (Bucar et al. 2013).

Prior to bioassay guided fractionation, the plant materials need to be extracted. In this chapter, the plant materials were extracted by a sequential extraction method using solvents of increasing polarity. The isolation of active fractions was done by employing two chromatography methods, namely, vacuum liquid chromatography (VLC) and

centrifugal thin layer chromatography (CTLC). The major classes of phytochemicals present in the extracts and fractions were then examined using chemical reagents. High performance liquid chromatography (HPLC) analysis was employed to study the chemical profile of the bioactive fractions.

2.1.1 Preparation of plant materials and extraction methods

Preparation of plant materials includes pre-washing, drying or freeze drying and grinding of plant materials. These steps enable a homogenous sample, with improved kinetics of analytic extraction while increasing contact of plant material's surface with the extraction solvent. Other necessary cautions, such as temperature should be taken into account to avoid loss of active constituents during plant material preparation (Sasidharan et al. 2011). Similarly if a plant is identified to contain volatile or thermolabile constituents, it is recommended to freeze the material right after collection. Frequently, collected plant materials are sliced into small pieces, then placed on trays and allowed to dry at moderate temperature with ventilation. While this procedure is widely practice, it is necessary to maintain a dry condition to avoid microbial contamination that potentially leads to metabolites degradation. Over exposure to sunlight should also be prevented as ultraviolet rays are capable of initiating chemical reactions that produce artifacts. Finally, the obtained dried material must be sealed in proper containers and kept in a dry and cool space (Seidel 2006).

Extraction is an important and crucial procedure in the study of medicinal plants as it has a great influence on the final outcome of the study (Azmir et al. 2013). Selection of suitable extraction method will ensure that reliable qualitative and quantitative studies of the isolated bioactive compounds will be obtained (Smith 2003; Sasidharan et al. 2011). A conventional method of extraction that depends on extracting capacity of different

solvent in combination of heat and/or mixing is still popular in natural product research. The methods can be categorized based on three different mechanical means, namely, soxhlet extraction, maceration and hydrodistillation (Azmir et al. 2013). Choices of solvents play a major part in extraction efficiency (Cowan 1999) because plant extracts exist as combination of various types of bioactive molecules of different polarities (Azmir et al. 2013). As such, polarity of the targeted compounds or group of compounds is a determinant factor for choice of extraction solvent. Table 4.1 represents different types of solvent that are used to extract selected group of plant metabolites.

Table2.1 Example of some bioactive compounds extracted by different solvents (adapted from Cowan 1999).

Water	Ethanol	Methanol	Chloroform	Dichloro-methanol	Ether	Acetone
Anthocyanins	Tannins	Anthocyanin	Terpenoids	Terpenoids	Alkaloids	Flavonoids
Tannins	Polyphenols	Terpenoids	Flavonoids		Terpenoids	
Saponins	Flavonol	Saponins				
Terpenoids	Terpenoids	Tannins				
	Alkaloids	Flavones				
		Polyphenols				

In this study, the plant materials were dried and grinded prior to extraction. Extraction of plant material was carried out by employing a sequential extraction approach from non-polar to polar solvents (hexane → ethyl acetate → ethanol) to exploit different solubility of plant matrices found in the extracts. Typically plant natural products can be separated into these categories; waxes and fatty acids, polyacetylenes, terpenoids, steroids, essential oils, phenolics, alkaloids, and glycosidic derivatives (Seidel 2006). Most fatty acids and essential oils can be extracted by non-polar solvents like hexane, while other components with mid-polarity (such as some alkaloids and flavonoids) are soluble in solvents such as ethyl acetate and chloroform, and more polar components (such as tannins and glycosides) can be extracted with ethanol and methanol. As such, application of selective extraction conducted sequentially with solvents of increasing polarity allows a

preliminary separation of the constituents of different polarity into distinct extracts and facilitate further purification/separation processes (Cottiglia et al. 2004; Seidel 2006).

2.1.2 Liquid chromatography methods

In natural product isolation, liquid chromatography (LC) represents the most common method for separating mixtures into their constituent compounds. It features a liquid mobile phase which dissolves the extract and runs the mixture through a solid stationary phase. Separation of the mixture into individual components or fractions is attributed to difference in the degree of binding of the solute molecules in the stationary phase (Dill 1987). The more commonly used LC involves the use of silica gel in an open column chromatography and a preparative thin layer chromatography (TLC) mainly because the two methods are rather straight forward with regards to the ease of use and relatively economical. Vacuum liquid chromatography (VLC) is a variant of LC and can be used to fractionate the crude extract swiftly, while centrifugal thin layer chromatography (CTLTC) is used for further fractionation and for purification of pure compounds (Hostettmann et al. 1998).

A VLC apparatus and specifications were designed to maintain the column in vacuum state to eliminate channelling issues. Application of the vacuum speeds up the eluent flow-rate. The method for VLC operation is fairly simple and it allows separation of large amount of extract up to 30 g per run. As such, VLC is often considered as technique that is efficient and rapid, and relatively inexpensive for organic compound separation (Sticher 2007). CTLTC was developed to overcome the problems of the many hours required to separate a mixture of compounds and the difficulties in removing the constituent substances from the plate of a conventional preparative TLC. A key feature of the method relies on the action of a centrifugal force to fasten mobile phase flow across

the circular TLC plate. The circular plate is made of glass plate covered with a layer of TLC grade silica sorbent with varying thickness of 1, 2 or 4 mm. An electric motor functions to rotate the plate at 800 rpm in which samples is loaded at the centre of the plate. Eluent will be pumped onto the centre of the plate, which subsequently flows across the sorbent due to centrifugal force. As a result, the sample will be separated into concentric bands across the plate that eventually elutes at the edge of the plate. These are collected and examined by using TLC analysis (Marston and Hostettmann 1991).

HPLC is an improvised version of column chromatography. It uses high pressure (up to 400 atmospheres) in contrast to vacuum or gravity in VLC/CTLC to force the solvent through a column. The main advantage of this technique is it uses much smaller particle size for the column packing materials hence, provides a larger surface area for interactions between the stationary phase and the molecules flowing through it. As a result, a better separation of the components in the mixture is achieved. This is coupled with an automated and sensitive detection method. One of the common methods is ultra-violet (UV) absorption. Organic compounds absorb UV lights at numerous wavelengths. When a mixture of compounds analyzed through HPLC, the output will be recorded as a series of peaks. Theoretically, each one peak represents a compound in the mixture passing through the UV detector (Clarke 2007).

Isolation and purification of fractions from the plant extracts in this research were achieved by using both the VLC and CTLC methods. In this study, normal phase silica gel was used as the stationary phase for VLC to fractionate the plant extracts into less complex fraction, which were further fractionated by using CTLC. Ethyl acetate-hexane/methanol was selected as the solvent system with gradual decrease of hexane followed by gradual increase of methanol. Whereas, HPLC methods were used to analyze

the complexity of the bioactive fractions by using solvent system comprising of acetonitrile (ACN)/miliQ water (H₂O).

2.1.3 Phytochemical analysis

The methods previously developed for phytochemical analysis are rather simple and straightforward, and can be easily performed in the laboratory (Sasidharan et al. 2011). The results are interpreted as negative or positive based on colorimetric changes that are achieved by using the relevant screening reagents. Since an extract contains a mixture of natural products, this method is effective for researchers to conjure hypotheses about the type of compounds present. In this chapter, phytochemical analysis was conducted on the crude extracts and bioactive fractions of *A. wilkesiana* and *D. grandiflora*. The screening methods were performed as described by Jones and Kinghorn 2005. The analysis was objectively done to detect major classes of phytochemicals, namely, tannins, alkaloids, flavonoids, sterols/steroids and glycosides.

2.2 AIMS AND OBJECTIVES

The specific objectives of this chapter are:

1. To identify major classes of compounds in extracts and fractions from *A. wilkesiana* and *D. grandiflora* by phytochemical analysis.
2. To determine fractions that has potent antimicrobial and at the same time is able to retain the activity throughout the studies via bioassay-guided fractionation.

2.3 MATERIALS AND METHODS

2.3.1 Plant collection and extraction

The plant material, *A. wilkesiana* was collected from Broga, Selangor, Malaysia (September, 2010) and *D. grandiflora* from Simpang Pulai, Perak, Malaysia (September, 2011). Voucher samples were deposited in the herbarium of Faculty of Science, University of Nottingham Malaysia Campus, where *A. wilkesiana* and *D. grandiflora* were assigned as UNMC 9 and UNMC 75, respectively.

The collected plant materials were dried and grinded prior to extraction. The dried plant materials (3.6 kg – *A. wilkesiana* whole plant, 2.1 kg – *D. grandiflora* leaves, 2.5 kg – *D. grandiflora* bark) were subjected to sequential extraction using n-hexane (He), followed by ethyl acetate (EA) and finally 95% ethanol (EtOH). Extraction in each solvent was conducted by soaking the plant material in the solvent (24 hours x 3 times) at room temperature. The plant material and extract solution were separated using a filter paper (Whatman Grade No. 1, USA). The filtrates were concentrated under reduced pressure at 35-40°C using a rotary evaporator (Buchi, USA). The concentrated crude extracts were further dried in a desiccator for 1-2 weeks as required. The dried crude extracts were kept in a -20°C freezer for further experiments.

2.3.2 Fractionation of crude extracts

The following crude extracts were fractionated in this study: 39.14 g of ethyl acetate extract of *A. wilkesiana* (9EA), 49.95 g of ethyl acetate leaf extract of *D. grandiflora* (75EA-L) and 16.36 g of ethyl acetate bark extract of *D. grandiflora* (75EA-B). Based on earlier finding in our laboratory, these extracts exhibited potent anti-staphylococcal activities

(Othman et al. 2011a) and were therefore chosen for bioassay-guided fractionation in the present study.

Crude extracts were fractionated by using a silica gel (40-63 microns, Mallinckrodt, USA) VLC (Figure 2.1). The solvent system used for elution was n-hexane (He) with increasing amount of chloroform (CHCl_3), and CHCl_3 with increasing amount of methanol (MeOH) [He/CHCl_3 (1:1) \rightarrow CHCl_3 \rightarrow $\text{CHCl}_3/\text{MeOH}$ (97:3 v/v) \rightarrow $\text{CHCl}_3/\text{MeOH}$ (95:5 v/v) \rightarrow $\text{CHCl}_3/\text{MeOH}$ (93:7 v/v) \rightarrow $\text{CHCl}_3/\text{MeOH}$ (90:10 v/v) \rightarrow $\text{CHCl}_3/\text{MeOH}$ (85:15 v/v)]. Fractions obtained were further fractionated via preparative CTLC (silica gel) using similar solvent systems (Figure 2.2).

TLC technique was used to monitor the extent of fractionation as well as to assist in combining fractions with similar profile (Appendix 7). The solvent systems used in TLC to combine the fractions were; $\text{CHCl}_3/\text{MeOH}$ 5%, CHCl_3/He (ratio 3:1, 2:1, 1:3 and 1:5), CHCl_3 100%, ethyl acetate (EA) 100%, diethyl ether (Et_2O)/MeOH 5%, EA/He (ratio 1:3 and 1:5). Fractions obtained were tested for anti-MRSA activity as described in Chapter 3 at every stage of fractionation and purification.

2.3.3 TLC analysis

Fractions that demonstrated potential anti-MRSA activity as described in Chapter 3 were analyzed using TLC methods to observe the complexity of these fractions and for chemistry profiling purposes. The solvent systems used for TLC were the same as described in section 2.3.2. In addition the following solvent systems were used; acetone (Ac) 100%, Ac/He (ratio 1:2), $\text{CHCl}_3/\text{MeOH}/\text{formic acid}$ (FA) (ratio 3:1:1), EA/MeOH 20%, $\text{CHCl}_3/\text{MeOH}$ 1%, 3%, 20%, 30% and 50%.

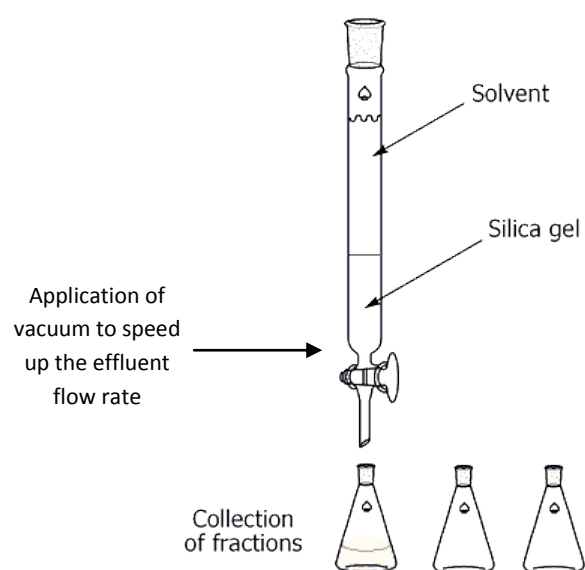


Figure 2.1 An example of a vacuum liquid chromatography (VLC) unit used for fractionation of plant crude extracts in this study (Image obtained from: <http://www.umich.edu/~chemh215/W11HTML/SSG2/ssg2.1/glossary1.html>).

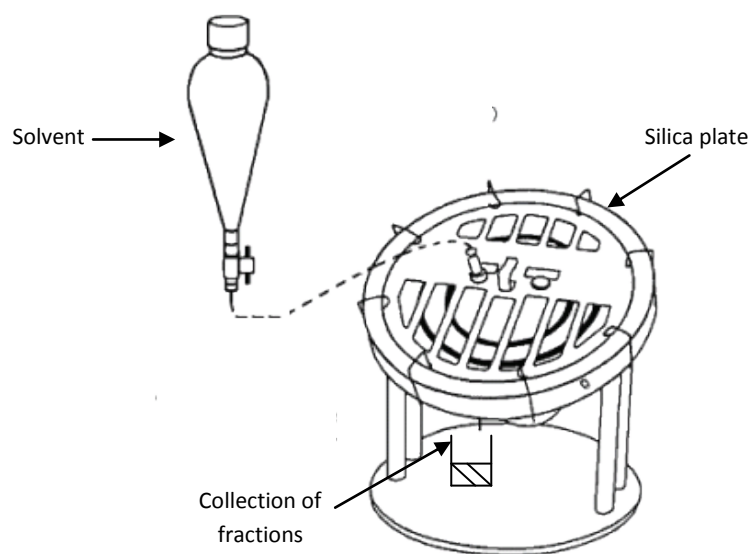


Figure 2.2 A diagram of a centrifugal thin layer column chromatography (CTLCC) unit used for separation of fractions obtained from vacuum liquid chromatography (VLC) (Image obtained from: <http://www.sbwave.com/chromatotron/specs.html>)

2.3.4 HPLC analysis

An aliquot of the selected bioactive fraction (40 µl of 10 mg/ml) was analyzed by C₁₈-reversed phase HPLC using the following gradient solvent system: 2 min at 10% acetonitrile (ACN)/miliQ water (H₂O); a linear gradient to 75% ACN/H₂O over 12 min; isocratic at 75% for 10 min; a linear gradient to 100% ACN for 2 min; isocratic at 100% ACN for 4 min. HPLC was performed on a Varian 940-LC system using a reversed phase analytical column (Pursuit XRs C18, 4.6 x 150 mm, 5 µm) with photodiode array (PDA) detection at 254 nm.

2.3.5 Determination of phytochemical contents

Phytochemical analysis was carried out according to the methods reported by Jones and Kinghorn 2005, as summarized below:

a) Tannins

200 mg of plant material was dissolved in 10 ml distilled water and filtered. 2 ml of filtrate was added to 2 ml of ferric chloride (5% w/v). The presence of tannins was indicated by blue black precipitation.

b) Alkaloids

200 mg plant material was dissolved in 10 ml methanol and filtered. 2 ml of the filtrate was added to acid hydrochloric (1% w/v) in boiling water bath. 1 ml of aliquot was transferred onto a wash glass and followed by addition of 6 drops of Dragendorff's reagent. A brownish/brown precipitation indicated presence of alkaloids in sample.

c) Flavonoids

200 mg plant material was dissolved in 10 ml ethanol and filtered. 2 ml of the filtrate was added to a test tube containing few drops of concentrated hydrochloric acid and magnesium ribbon. Detection of flavonoids was confirmed when the solution turns into pink-tomato red colour.

d) Saponins

0.5 ml of the plant filtrate was added into 5 ml of distilled water and the mixture was shaken. Formation of frothing which persisted for 15 minutes after the shaking indicated the presence of saponins.

e) Sterols/Steroids

1 mg of plant material was dissolved in 1 ml of CHCl_3 . This was followed by addition of 1 ml of concentrated sulfuric acid. The presence of sterols/steroids was indicated by the formation of red colour in the solution.

f) Glycosides

2 ml of plant filtrate was added to 1 ml glacial acetic acid in the presence of ferric chloride and concentrated sulfuric acid. The presence of glycosides was indicated by green-blue colour in the solution.

2.4 RESULTS

2.4.1 Extraction of UNMC 9 and UNMC 75

Both the UNMC 9 and UNMC 75 plant materials were sequentially extracted with n-hexane, ethyl acetate and ethanol. The percentage yield of each extraction is summarized in Table 2.2. Based on the results, extraction using ethanol afforded the highest yields. The leaves of UNMC 75 also afforded higher yields compared to the bark.

Table 2.2 Results of sequential extraction of UNMC 9 and UNMC 75.

Types of extracts	Plant parts	Weight (g)	Yield (%)
9EtOH	Whole plant	266.20	7.6
9EA	Whole plant	71.77	2.1
9He	Whole plant	70.00	2.0
75EtOH-L	Leaves	101.90	4.9
75EA-L	Leaves	79.31	3.8
75He-L	Leaves	81.19	3.9
75EtOH-B	Bark	92.33	3.7
75EA-B	Bark	18.90	0.8
75He-B	Bark	Not determined	-

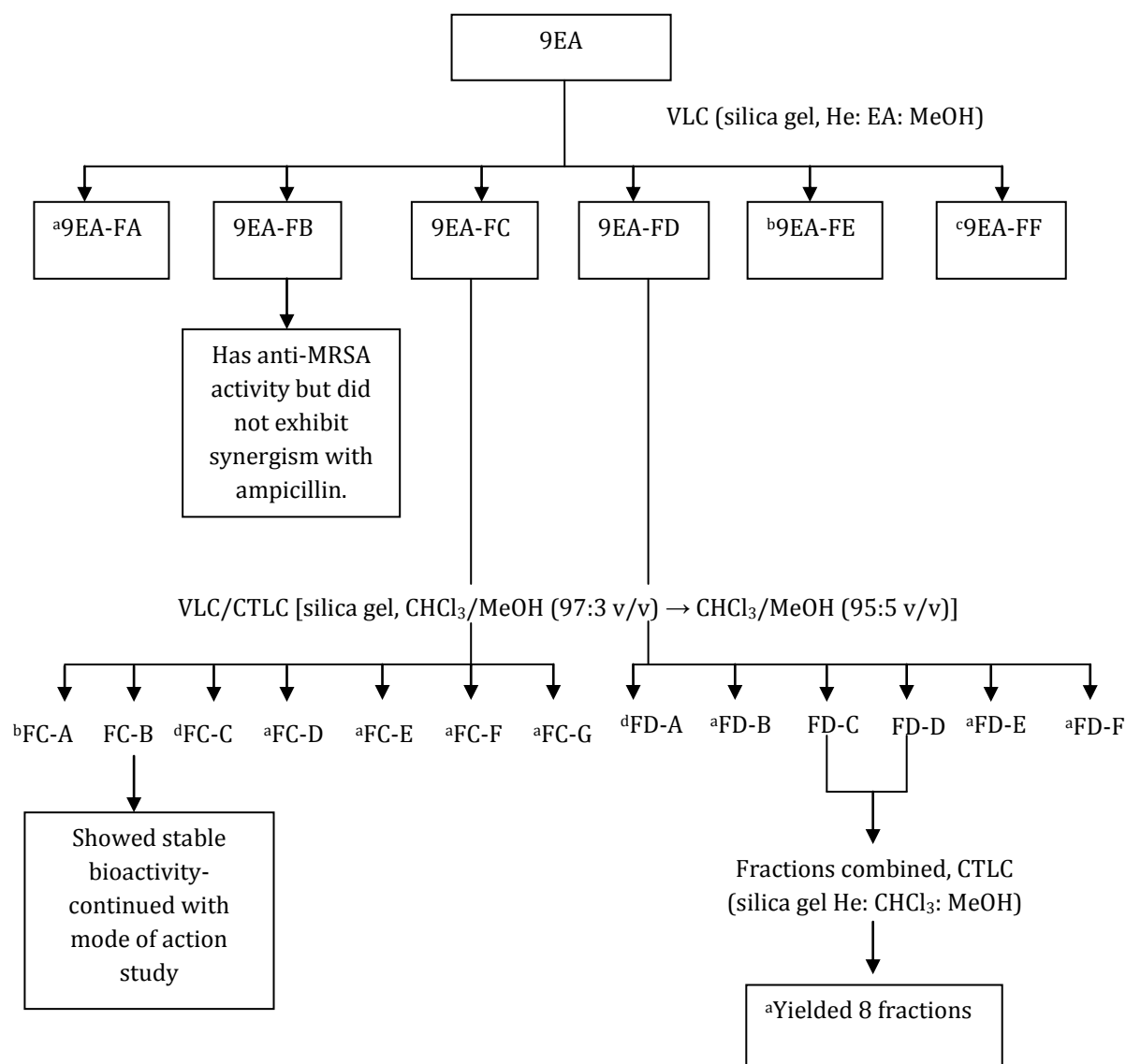
2.4.2 Bioassay guided fractionation of 9EA, 75EA-L and 75EA-B

The three ethyl acetate crude extracts, namely 9EA, 75EA-L and 75EA-B, were first fractionated by VLC and followed by CTLC. The fractions obtained from VLC and CTLC were combined based on their TLC profiles (Appendix 7). At each stage of the fractionation, the obtained fractions were tested for anti-MRSA activity. Only fractions that showed the desired bioactivity were further fractionated

VLC of 9EA afforded six major fractions (Figure 2.3), namely 9EA-FA, 9EA-FB, 9EA-FC, 9EA-FD, 9EA-FE and 9EA-FF. All fractions demonstrated anti-MRSA activity (section 3.4.3, Chapter 3) except 9EA-FA. Table 2.3 is showing solvent system which eluted the major fractions from 9EA.

Out of these, two fractions 9EA-FC and 9EA-FD were further fractionated. 9EA-FB although exhibited anti-MRSA activity, the fraction did not demonstrate any synergism with ampicillin. Hence, 9EA-FB was not selected for the subsequent fractionation process. Whereas, fraction 9EA-FE and 9EA-FF were insufficient in amount and highly polar respectively, thus preventing further works.

Fractionation of 9EA-FC and 9EA-FD on CTLC yielded 21 sub-fractions which were considered semi-pure fractions. Many of these semi-pure fractions suffered significant loss of bioactivity. An unstable bioactivity was noted in one of the semi-pure fraction FC-C, in which we saw the anti-MRSA activity decreased after being tested several times on different occasion (results not shown). Eventually, only fraction FC-B demonstrated the desired biological activity and was chosen as suitable candidate for further experiments.



Note: ^a –not active, ^b-insufficient amount for further isolation, ^c-highly polar fraction flushed with ethanol from the column, ^d- unstable activity

Figure 2.3 Schematic diagram of bioassay guided fractionation and purification of 9EA.

Table 2.3 Solvent system used for isolation of major fractions from 9EA on VLC.

Fractions	Solvent system
Crude extract 9EA	EA 100%
9EA-FA	He/CHCl ₃ (ratio 1:1)
9EA-FB	CHCl ₃ (100%) to CHCl ₃ /MeOH 1%
9EA-FC	CHCl ₃ /MeOH 1-5%
9EA-FD	CHCl ₃ /MeOH 5-7%
9EA-FE	CHCl ₃ /MeOH 10-15%
9EA-FF	EtOH 100%

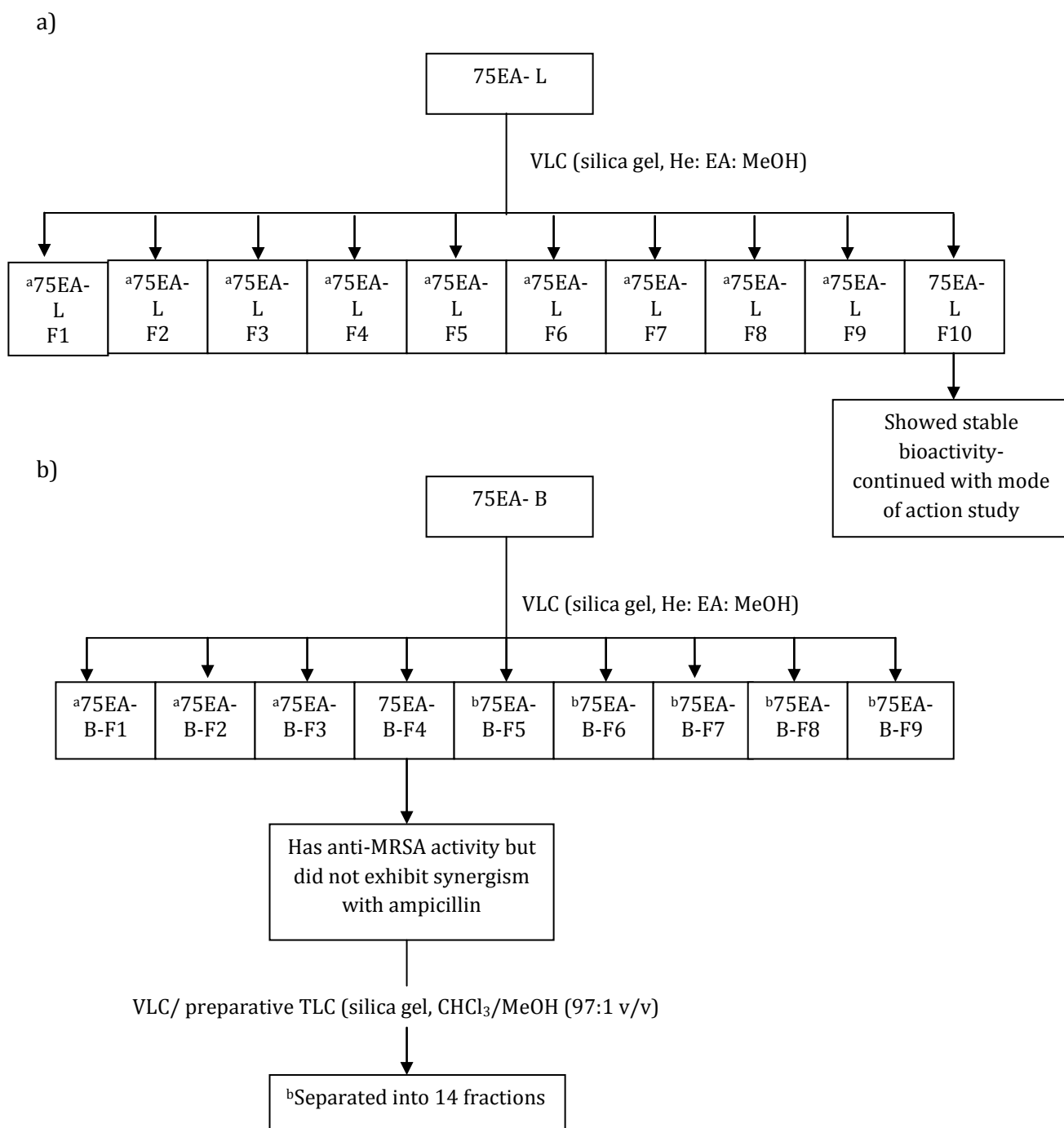
Fractionation of 75EA-L (Figure 2.4) on VLC resulted in 10 major fractions. Table 2.4 shows the solvent system in which the major fractions were eluted. All these major fractions were devoid of anti-MRSA activity except fraction 75EA-L-F10. Due to the very polar nature of 75EA-L-F10, this fraction was not fractionated further and was subjected to further biological study since it showed potent antimicrobial activity. In the case of 75EA-B, six out of nine of the sub-fractions were active in anti-MRSA test. The solvent system in which these fractions were eluted is shown in Table 2.5. However, five of these were eliminated from further study due to poor yield (< 500 mg). This brought us down with one fraction, namely 75EA-B-F4, which only showed anti-MRSA activity but not synergism with ampicillin. We carried out VLC/ CTLC on 75EA-B-F4 that gave rise to 14 semi-pure fractions. Nevertheless, the low amount of these fractions prevented further chemical analysis and biological evaluation.

Table 2.4 Solvent system used for isolation of major fractions from 75EA-L on VLC.

Fractions	Solvent system
Crude extract 75EA-L	EA 100%
75EA-L-F1	He/CHCl ₃ (1:1)
75EA-L-F2	CHCl ₃ 100%
75EA-L-F3	CHCl ₃ /MeOH 1%
75EA-L-F4	CHCl ₃ /MeOH 1-3%
75EA-L-F5	CHCl ₃ /MeOH 4-5%
75EA-L-F6	CHCl ₃ /MeOH 5-7%
75EA-L-F7	CHCl ₃ /MeOH 7-10%
75EA-L-F8	CHCl ₃ /MeOH 7-10%
75EA-L-F9	CHCl ₃ /MeOH 10-15%
75EA-L-F10	EtOH 100%

Table 2.5 Solvent system used for isolation of major fractions from 75EA-B on VLC.

Fractions	Solvent system
Crude extract 75EA-B	EA 100%
75EA-B-F1	He/CHCl ₃ (1:1)
75EA-B-F2	He/CHCl ₃ (4:1)
75EA-B-F3	CHCl ₃ /MeOH (1%)
75EA-B-F4	CHCl ₃ /MeOH (1-3%)
75EA-B-F5	CHCl ₃ /MeOH (4-5%)
75EA-B-F6	CHCl ₃ /MeOH (5-7%)
75EA-B-F7	CHCl ₃ /MeOH (7-10%)
75EA-B-F8	CHCl ₃ /MeOH (20%)
75EA-B-F9	EtOH (100%)



Note: ^a –not active, ^b-insufficient amount for further isolation, ^c- fraction flushed with ethanol from the column, ^d- unstable activity

Figure 2.4 Schematic diagram of bioassay guided fractionation and purification of a) 75EA-L and b) 75EA-B.

2.4.3 HPLC analyses of the selected bioactive fractions from 9EA and 75EA

Two bioactive fractions (9EA-FB and FC-B) from 9EA were analyzed via HPLC. These fractions were selected because 1) 9EA-FB demonstrated the most potent anti-MRSA and anti-MSSA activity based on the lowest MIC value and 2) FC-B showed anti-MRSA activity and synergism with ampicillin (section 3.4.2 and section 3.4.6, Chapter 3). Figure 2.5 shows chromatograms of the respective bioactive fractions and the results revealed a complex series of peaks belonging to mixture of the compounds in the fractions indicative of incomplete separation of the compounds.

From 75EA, two bioactive fractions (75EA-B4 and 75EA-L-F10) were analyzed using HPLC. 75EA-L-F10 was chosen because it was the only fraction that displayed anti-MRSA activity in addition to synergism with ampicillin (section 3.4.3 and 3.4.6, Chapter 3). Fraction 75EA-B-F4 whereas, exhibited a strong inhibition on MRSA and MSSA with the lowest MIC value (section 3.4.3, Chapter 3). The results are shown in Figure 2.6. Similar to bioactive fractions from 9EA, these two fractions were also observed to comprise of multiple overlapping peaks showing incomplete separation of compounds in the mixture. Fraction 7EA-B-F4 although it consist several peaks, overall, the chromatogram appeared less complex compared to other fractions. Whereas, peaks belonging to mixture of compounds in 75EA-L-F10 have low intensity (< 1000 mAU) in contrast to other analyzed bioactive fractions.

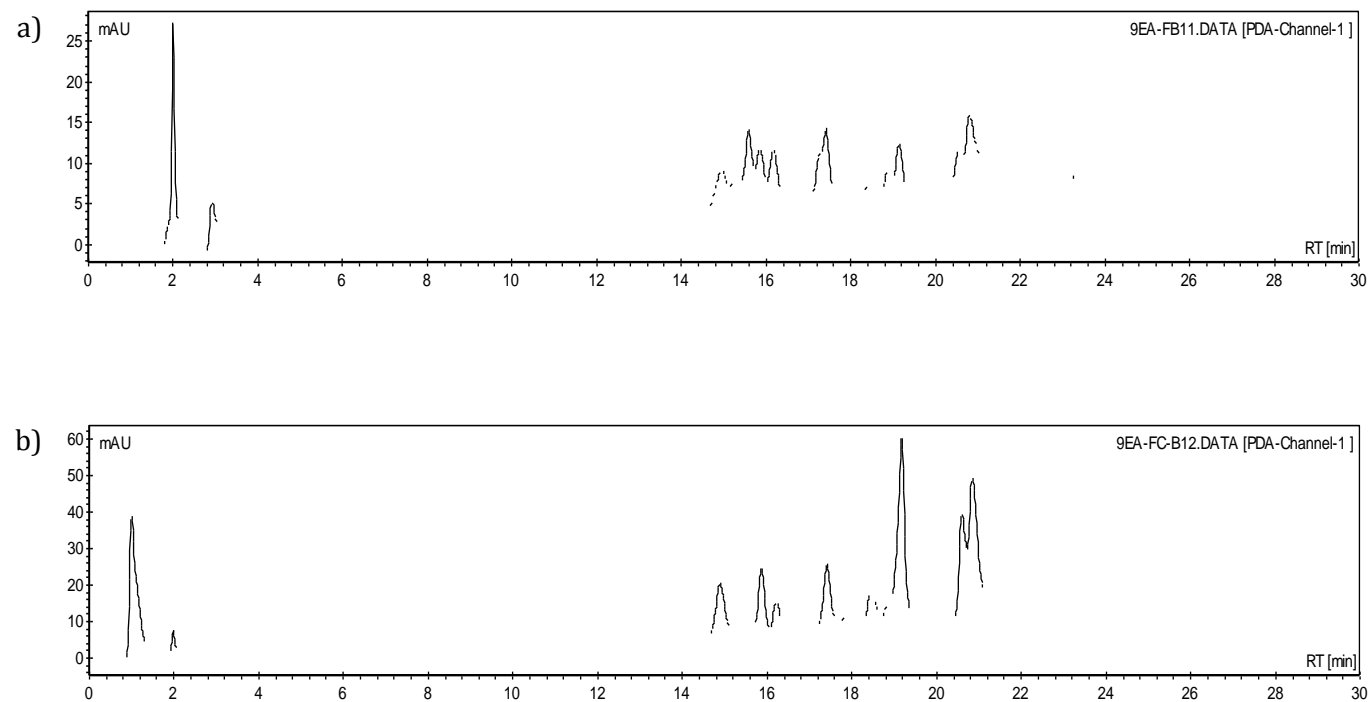


Figure 2.5 HPLC analyses of a) 9EA-FB and c) FC-B. An aliquot of 40 μ l of 10 mg/ml of each fraction analyzed by C_{18} -reversed phase (4.6 x 150 mm, 5 μ m) detected at 254 nm.

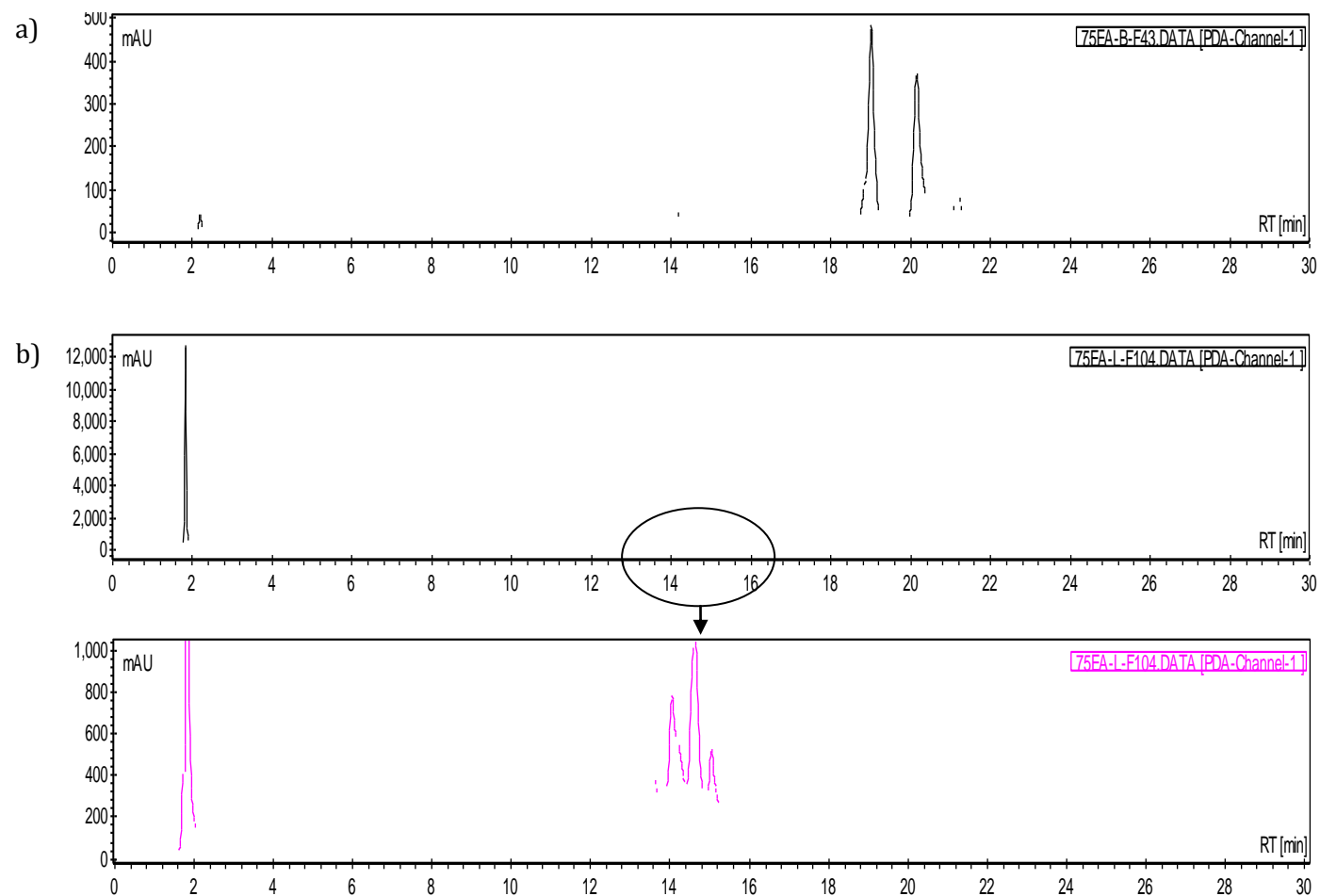


Figure 2.6 HPLC analyses of a) 75EA-B-F4 and b) 75EA-L-F10. An aliquot of 40 μ l of 10 mg/ml of each fraction analyzed by C_{18} -reversed phase (4.6 x 150 mm, 5 μ m) detected at 254 nm.

2.4.4 TLC profiles of selected bioactive fractions from 9EA and 75EA

Similar to section 2.4.3 (HPLC analyses), the same bioactive fractions identified from 9EA and 75EA were analyzed via TLC method. The bioactive fractions were 9EA-FB, FC-B, 75EA-L-F10, and 75EA-B-F4. In general, the TLC profiles showed presence of multiple spots representing mixture of compounds occurring in the respective fractions. Figures 2.7 to 2.10 are showing a TLC profiles for these fractions accessed in various solvent systems in which these fractions were separated. The solvent system Ac/He (ratio 1:2) was found to result in well separation of the components in all the fractions except 75EA-L-F10. In addition, TLC profiles of 9EA-FB (Figure 2.7) and FC-B (Figure 2.8), indicated that these two fractions were likely to contain similar compounds. Also, we found components in 75EA-L-F10 did not separate into distinct spots unlike other tested fractions but rather formed a long streak along the TLC plate (Figure 2.10).



Figure 2.7

TLC profile of fraction 9EA-FB isolated from crude extract *A. wilkesiana* analyzed in the solvent system (from far left) CHCl_3 100%, $\text{CHCl}_3/\text{MeOH}$ 1%, and Ac/He (ratio 1:2).

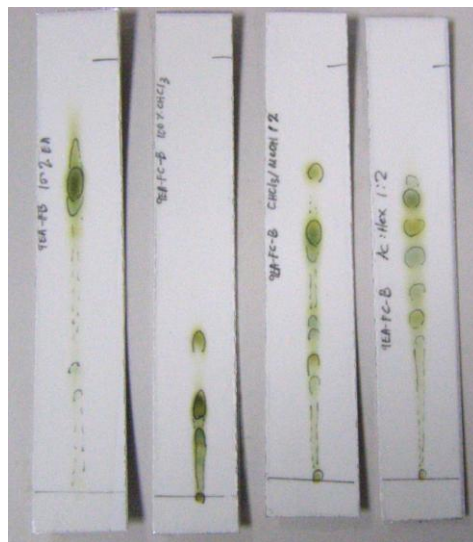


Figure 2.8 TLC profile of fraction FC-B isolated from crude extract *A. wilkesiana* analyzed in the solvent system (from far left) EA 100%, CHCl_3 100%, $\text{CHCl}_3/\text{MeOH}$ 1%, and Ac/He (ratio 1:2).



Figure 2.9 TLC profile of fraction 75EA-B-F4 isolated from crude bark extract *D. grandiflora* analyzed in the solvent system (from far left) EA 100%, CHCl_3 100%, $\text{CHCl}_3/\text{MeOH}$ 1%, $\text{CHCl}_3/\text{MeOH}$ 3%, $\text{CHCl}_3/\text{MeOH}$ 5%, and Ac/He (ratio 1:2).



Figure 2.10 TLC profile of fraction 75EA-L-F10 isolated from crude leaf extract *D. grandiflora* analyzed in the solvent system (from far left) Ac 100%, Et₂O/MeOH 5%, CHCl₃/MeOH/FA (ratio 3:1:1), EA/MeOH 20%, CHCl₃/MeOH 50%, CHCl₃/MeOH 20%, and CHCl₃/MeOH 30%.

2.4.5 Major classes of compounds in extracts and fractions from 9EA and 75EA

Phytochemical analysis was carried out on the crude extracts of 9EA, 75EA-L and 75EA-B and selected fractions that demonstrated anti-MRSA and synergism with ampicillin, (section 3.4.3 and section 3.4.6, Chapter 3). The analysis revealed the presence of commonly occurring classes of phytochemicals such as tannins, saponins, sterols/steroids, and glycosides. Classes of compounds that are frequently associated with biological activities such as alkaloids and steroids were also found in the extracts/fractions. Table 2.6 shows the various phytochemicals detected in the extracts/fractions.

Table 2.6 Phytochemical analysis of crude extracts and fractions from 9EA, 7EA. Experiments were conducted three times on separate occasions.

Phytochemicals	Extracts/ Fractions								
	9EA	9EA-FB	9EA-FC	9EA-FD	FC-B	75EA-L	75EA-B	75EA-L-F10	75EA-F-B4
Tannins	+	+	++	++	++	++	+	+	+
Alkaloids	+	-	+	++	-	+	+	-	-
Flavonoids	-	-	-	-	-	+	+	+	+
Saponins	+	+	+	+	+	+	+	+	+
Sterols/Steroids	+	++	++	++	++	++	+	+	++
Glycosides	+	++	++	+++	++	++	+	++	++

Note: - absent, + present, ++ moderate amount, +++ appreciable amount

2.5 DISCUSSION

Natural products have been identified as an important mainspring of novel structures particularly in anti infective research since the research activities highly depends on the structural information of these products for development of new drugs. Although application of combinatorial chemistry improves activities of newly approved agents, so far in the time frame of 30 years, only one compound optimized from this technique was approved as a drug by FDA. The approved compound is an anti tumor agent known as sorafenib (Nexavar®) from Bayer, for treatment of renal cell carcinoma. This shows that the study of natural products continue to play a vital role in the discovery and development of drugs (Newman and Cragg 2012).

Our study in this chapter employed bioassay guided fractionation method to uncover the active fractions from the extracts 9EA and 75EA with anti-MRSA activity. The fractionation process combined with biological assays has enabled the identification of two main bioactive fractions, FC-B and 75EA-L-F10, which possessed improved anti-MRSA activity compared to the corresponding crude extracts. The two fractions demonstrated synergism with ampicillin in suppressing MRSA growth in addition to retaining the bioactivity throughout this study (Chapter 3). A study on phytochemical contents in the crude extracts and bioactive fractions used in this chapter was conducted and the results showed the presence of biologically active classes of phytochemicals such as tannins, alkaloids, flavonoids, saponins, glycosides and steroids. In agreement, the HPLC analyses revealed occurrence of mixture of phytochemicals represented by severe overlapping of peaks observed in the chromatograms of the bioactive fractions.

2.5.1 Bioassay guided fractionation

In the present study, the crude extract 9EA was separated into major six major fractions. Based on evaluation of MIC values against MRSA and MSSA (Chapter 3), all fractions except 9EA-FA exhibited antimicrobial activity. Two out of the five major fractions, namely, 9EA-FC and 9EA-FD, were selected for further purification due to their stable activity and sufficient yields for further testing. VLC/CTLC of 9EA-FC and 9EA-FD resulted on the isolation of seven and six semi-pure fractions, respectively. A prominent pattern among these semi-pure fractions was they were biologically inactive when tested. Only one semi-pure fraction, FC-B was identified to exhibit the desired anti-MRSA activity. These results indicated that bioactivity for most of the semi-pure fractions were abolished following the separation process. A similar trend was observed amongst the major fractions isolated from 75EA-L,

where only one (75EA-L-F10) out of 10 isolated fractions displayed good anti-MRSA activity. On the other hand, 75EA-B gave rise to a total of nine major fractions after VLC. Further purification of fraction 75EA-B-F4 yielded 14 semi-pure fractions. However, the very low amount of the fractions obtained did not permit further antimicrobial work to be carried out.

Based on the results described above, the major issue faced during the bioassay guided fractionation was loss of bioactivity with subsequent separation or purification. Loss of bioactivity after fractionation was apparently a common phenomenon observed during isolation of active components from plant extracts. A possible reason for loss of bioactivity observed in this study can be linked with synergism effects between occurring compounds in an extract which collectively contributed to the biological activity. In phytomedicine, it is common to utilize whole plant rather than a single compound which suggests that compounds in the extract may work in concert to produce the observed biological activity. In fact, a previous study concluded that synergism should be anticipated when an extract loss its initial bioactivity after fractionation (Williamson 2001; Rasoanaivo et al. 2011). Researches from the past have published *in vitro* and other experimental evidence demonstrating synergistic effects amongst compounds in an extract and lapsing of the bioactivity that occurred following isolation of compound (Singh and Blumenthal 1997; Houghton 2000; Rasoanaivo et al. 2011). As an example, a study conducted by Houghton (2000) saw that fractionation of *Kigelia pinnata* extract had eradicated the initial cytotoxic effects observed. Apart from the synergism theory, another possibility would be that the bioactive constituent might be unstable on its own and needs the presence of other compounds such as antioxidant to protect it from decomposition (Williamson 2001). Thus, the isolation process may have eliminated bioactivity due synergism between the extract's components or compromised the stability of the active ingredients. This may be the possible

explanations for the poor and unstable anti-MRSA activity observed for the semi-pure fractions derived from 9EA and 75EA.

2.5.2 Occurrence of phytochemicals in crude extracts and bioactive fractions of *A. wilkesiana* and *D. grandiflora*

In phytochemical analysis, 9EA, 75EA-L and 75EA-B together with their respective fractions were found to contain various classes of plant natural products such as tannins, alkaloids, flavonoids, saponins, sterols/steroids, and glycosides. These phytochemicals has been previously identified as potential drug candidates. The advantage of identifying the types of phytochemicals present in a plant extract or fraction provides useful information on possible mode of action for the observed biological activities (Cowan 1999; Saleem et al. 2010).

The extract 9EA is from the plant *A. wilkesiana* which has been studied by others earlier (Adesina et al 2000; Oladunmoye 2010; Akinyemi et al. 2005; Othman et al. 2011a). Preceding studies have reported frequent occurrence of tannins and saponins in *A. wilkesiana* extracts. Likewise, in this study, high concentrations of tannins were detected in 9EA and in the major fractions, 9EA-FC and 9EA-FD. Compounds belonging to the tannin group such as corilagin, ethyl gallate, ellagitannin and geraniin were observed to exhibit antibacterial activities, specifically against *S. aureus* sp. (synergism with beta-lactams were reported) (Shimizu et al. 2001; Sato et al. 2004; Din et al. 2013a, Din et al. 2013b). The mechanism of actions is thought to be related to the ability of tannins to lyse bacterial cells which eventually causes cell death (Adesina et al. 2000; Oladunmoye 2010). Furthermore, tannins like epigallocatechin-gallate and corilagin were found to suppress resistant factors

in MRSA such as production of beta-lactamase and production or activity of PBP2a (Hu et al. 2001; Shimizu et al. 2001). Hence the presence of tannins in the extract 9EA and fractions, derived from it may explain the good antimicrobial activity displayed by the extract/fractions against MRSA (Chapter 3).

The extracts 75EA-L and 75EA-B from *D. grandiflora* along with the bioactive fractions obtained from these extracts were tested positive for all the phytochemicals except alkaloids which was absent in the bioactive fractions obtained from these crude extracts. The results are identical with an earlier study conducted by Othman et al. (2011b) which also showed presence of similar phytochemicals in *D. grandiflora* extracts that possess anti-staphylococcal activities. Literature review on this plant's antimicrobial activity is very limited. Thus, although the anti-MRSA activity observed in this study is attributed to the presence of phytochemicals found in extracts/fractions, we are unable to deduce which class of phytochemicals are more likely to contribute to the observed antimicrobial activity.

CHAPTER 3

ANTIMICROBIAL ACTIVITY OF PLANT EXTRACTS AND FRACTIONS

3.1 BACKGROUND

In the late 1990s, the usage of plant extracts and other alternative forms of plant products have become popular to treat or prevent infectious diseases. This is because the public was becoming more conscious of problems associated with over prescription and exploitation of traditional antibiotics (Cowan 1999). These herbal medicines and supplements were perceived to be safe and do not have much side effects in comparison to synthetic drugs (Iniaghe et al. 2009). Following that, natural product chemists and microbiologist concentrate in exploration on medical plants as they suspect the wide range of plants accompanied by their chemically diverse phytochemicals may have unique mechanism in combating antibiotic-resistant pathogens. As such, these plant-derived metabolites may serve as an adjuvant or provide new leads for alternative antibacterial agents (Abreu et al. 2012).

MRSA particularly has been selected for this study as it is known as the major cause of nosocomial infections and results in complications in many cases which eventually leads to death (Aqil et al. 2005). A review by Livermore (2009) stated available treatment options for MRSA infections are expanding with usage of drugs such as daptomycin, linezolid and tigecycline. However, the level and complexity of resistance mechanism displayed by this pathogen is evolving simultaneously due to increased uncontrolled utilization of antibiotics (Snider and Rivard 2012). Resistance in MRSA is mainly attributed to acquisition of *mecA* gene which confers resistance to beta-lactam antibiotics. The gene encodes for production of PBP2a, an altered protein that evades antimicrobial action of beta-lactams due to its low binding affinity. PBP2a replaces the function of normal PBPs which are susceptible to beta-lactams (Berger-Bächi and Rohrer, 2002; Deresinski, 2005). As such, the bacterial cell

biosynthesis which is the target of beta-lactam's action is not compromised upon challenged by these drugs. This creates the need to find new agents that are able to counteract the mechanism of resistance displayed by MRSA from further evolving. One of the proposed approaches in overcoming bacterial resistance is synergism by combining constituents from a plant extract with drugs to achieve a better therapeutic efficacy (Hemaiswarya et al. 2008; Wagner and Ulrich-Merzenich 2009).

In this chapter, we have embarked on the investigation of two medicinal plants; *A. wilkesiana* and *D. grandiflora*. We focused on ethyl acetate extracts based on findings from an earlier study (Othman et al. 2011a). The crude ethyl acetate extracts of these plants were fractionated by using a bioassay-guided isolation method (Chapter 2) which managed the isolation of major fractions and semi-pure fractions that were used for this study. The first section of this chapter describes broth microdilution assay that was conducted to determine MIC of crude ethyl acetate extracts and fractions from both plants against MRSA. The second part describes the synergistic studies which were carried out on combinations of plant test agents and ampicillin via two experiments; kinetic growth curve experiment and fractional inhibitory concentration (FIC) index interpretation. The third section is investigation of mode of action of the selected combination treatments. This was carried out by employing Western blot experiment to study expression of PBP2a.

Selection of bioactive fractions for the combination studies was determined from their MIC results. In this chapter, experiments were conducted on fractions from three different extracts 1) ethyl acetate extract of *A. wilkesiana* (9EA), 2) ethyl acetate extract of *D. grandiflora* leaves (75EA-L) and 3) ethyl acetate extract of *D. grandiflora* bark (75EA-B). These three crude extracts initially evaluated for anti-MRSA activity via MIC determination.

Subsequent purification processes (LC) have separated these crude extracts into major and semi-pure fractions. Since, the approach in selecting bioactive fraction in this research is bioassay-guided; fractions were evaluated for anti-MRSA activity every time after each step of purification. Fractions that retained anti-MRSA activity after the purification were investigated for any synergistic effects with ampicillin. Table 3.1 lists the fractions reported in this chapter.

Table 3.1 Selected extracts and fractions of *A. wilkesiana* and *D. grandiflora* for combinatory studies (synergistic).

Plant	Code	Extract type
<i>A. wilkesiana</i>	9EA	Crude extract
	9EA-FB	Major fraction
	9EA-FC	Major fraction
	9EA-FD	Major fraction
	FC-B	Semi-pure fraction
<i>D. grandiflora</i>	75EA-L	Crude extract
	75EA-L-F10	Major fraction

Note: The crude extract 75EA-B and its fractions were not selected for this study due to insufficient amount for the testing

Bioactive fractions which demonstrated synergism with ampicillin in the combination treatments were selected for study on mechanism of action in which their effects on PBP2a expression were investigated. Two fractions reported in the Table 3.1, FC-B and 75EA-L-F10 were chosen based on their ability to reduce MIC of ampicillin the most and successfully inhibited MRSA growth in the combinatory studies. The studied combination treatments were i) $1/32 \times \text{MIC ampicillin} + 1/4 \times \text{MIC FC-B}$ and ii) $1/64 \times \text{MIC ampicillin} + 1/4 \times \text{MIC 75EA-L-F10}$.

3.1.1 Bacterial strains and growing conditions

The two *S. aureus* strains used in this study (MRSA ATCC 43300 and MSSA ATCC 11632) have been fully characterized and they were purchased commercially from Microbiologics (USA). The strain MRSA ATCC 43300 confers resistance to oxacillin, methicillin, ampicillin, and penicillin and has been used as a reference strain in many previous studies (Tiwari and Sen 2006; Mataraci and Dosler 2012; Chung et al. 2013). The strain is also confirmed to be carrying the *SCCmec* chromosome with *mecA* gene regulator that is responsible for the resistance to the whole class of beta-lactams (Petrelli et al. 2008). MSSA ATCC 11632 strain on the other hand, is susceptible to oxacillin, methicillin and ampicillin, and served as a non-resistance strain control in our study. It has been used as a susceptible control in other screening assays and was reported as a *mecA* negative strain indicating absence of resistance towards to beta-lactams (Bekkaoui and Cloney 2003; Din et al. 2013a).

Factors such as type of media, supplements, growth temperature, and incubation duration affect the expression of methicillin resistance in *S. aureus*. Clinical Laboratory Standards Institute (CLSI) requires usage of Mueller-Hinton (MH) broth plus 2% sodium chloride (NaCl) in laboratory diagnosis and susceptibility testing of MRSA (CLSI 2007). MH media has been also recommended by European Committee on Antimicrobial Susceptibility Testing (EUCAST) since it is a general purpose medium that is employed to grow a wide range of non-fastidious microorganisms (EUCAST 2003). Affirmatively, older research reported that MRSA was more distinguishable on MH media (Brown and Kothari 1974; Monsen et al. 2003) and a better detection of methicillin resistance was obtained when the media is supplemented with NaCl (Monsen et. al. 2003). Accurate detection of methicillin resistance can be challenging due existence of sub-population within a culture. This is

because cells grow in the same culture carried different genetic information for resistance, and only a few cells may express resistance *in vitro* (Chambers 1988; Brown 2001). Since MRSA strains possess salt tolerant capabilities, NaCl (up to 5%) is added to the growth media as a selective agent and this improves sensitivity for culturing of MRSA. In this study, 2% of NaCl was added to the growth media as recommended by CLSI for MRSA testing (CLSI 2007). Although some studies suggested 2.5% of NaCl to be used for optimal sensitivity, higher salt concentrations may inhibit bacterial growth (Bruins et al. 2007).

Temperature and incubation duration employed in these experiments were stringently followed according to CLSI methods for MRSA testing. According to the institution's supplement document (M100-S17), duration of 24 hours incubation at 33-35°C is defined as the only method in general use for broth dilution assays (CLSI 2007). Due to heterogeneous resistant nature of the MRSA strains, extended incubation time and lower growth temperature were employed (Skov et al. 2009). An extended incubation hour is necessary because some heterogeneous strains grow very slowly; hence the test should be incubated for 24 hours rather than 16-20 hours as in other bacteriological tests (Brown et al. 2005; Andrews 2009). With regards to growth temperature, lower temperature is more favorable in detecting resistance. In 1970, Parker and Hewitt (1970) reported that a MRSA strain was highly resistant to the tested antibiotic at a lower temperature, and at 37°C the same strain grew and behaved like a normal staphylococcal strain. A more recent study, recommended that a growth temperature not exceeding 35°C should be used as false-negative results for resistance was observed for MRSA strains incubated at 36°C (Skov et al. 2009). The growth conditions for MRSA and MSSA in this study are described in section 3.3.1.

3.1.2 Broth microdilution assay

A common choice of assay used to evaluate the antibacterial actions of plant extracts is using the broth microdilution assay to determine the MIC (Ncube et al. 2008). MIC is interpreted as the lowest concentration of an antimicrobial agent that inhibits visible bacterial growth after overnight incubation (Andrews 2001). The determination of MIC is useful in monitoring susceptibility of an organism to a specific antibacterial compound (EUCAST 2003). Typically after an overnight incubation, the bacterial growth becomes visible and detected by turbidity in the broth. This is normally done to determine an antibiotic's MIC. Plant extract or fractions however are normally colored and cloudy. This may interfere with broth's turbidity reading. Hence, usage of colometric indicators will aid the interpretations of MIC breakpoints by eliminating ambiguity related with visual comparison (Ncube et al. 2008).

Advantages of broth microdilution method include testing of large samples and with a wider range of concentration compared to other methods such as diffusion techniques. It also has advantages as the method requires small quantities of extract/fraction for testing and this is particularly useful in natural product research due to a common problem of scarcity of fraction or pure compound (Langfield et al. 2004). In laboratory practice, broth microdilution method is conducted by preparing series of two-fold dilution to create a concentration range. Usually a range of 5 to 8 different concentrations are tested. Then, a standardized bacterial inoculums suspension is added into each well and incubated at appropriate temperature to determine the final MIC value.

An important criterion in designing protocol for MIC determination is the test bacteria. The bacteria should be isolated in pure culture and identified before subjecting it for the test. Standardization of bacterial inoculum size is crucial for obtaining precise and reproducible results. An inoculum size of 5×10^5 colony-forming units (CFU)/ ml is reported as the suitable cell number for this assay (EUCAST 2003; Cos et al. 2006; CLSI 2007; Wiegand et al. 2008). Higher or lower size of inoculums is likely to result in inaccurate MIC, especially in testing of resistant strain (Cos et al. 2006). In preparing the bacterial inoculums, a fresh pure culture should be used. To ensure all cells contain same genetic material, one must culture four-five single colonies of the bacteria to prepare a bacterial suspension, and incubate to reach density of 10^8 CFU/ml for inoculation in the assay (Cos et al. 2006; Wiegand et al. 2008, Othman et al. 2011b). Another option is direct colony suspension into liquid (Wiegand et al 2008). The prepared bacterial suspension must be used within 30 minutes to avert changes in cell numbers (EUCAST 2003).

Besides bacterial suspension, preparation of plant extract or fraction is of critical importance. A stock solution of extract is obtained in solvent such as methanol, acetone and dimethyl sulfoxide (DMSO) because besides having ability to completely dissolve the extract, they do not inhibit the microorganism at a cut of point of 2% in final concentration (Mathekga et al. 2000; Baris et al. 2006; Ncube et al. 2008). Similarly, antibiotics solution too has to be prepared accordingly to be included as quality control. Antibiotics were suggested to be prepared freshly by dissolving them in sterile distilled water to obtain stock solution. Other precautions such as storing the antibiotic in the dark containing desiccants and using potency information to formulate antibiotics solutions are equally important (Andrews 2001). Generally in preparing both antimicrobial agents (plant extract and antibiotics) the following measures should be applied; warming the agents at room temperature at least 2

hours before weighing to avoid condensation on the powder, using sterile spatula and containers to weigh out, containers used are cold resistant, sealed tightly (e.g. Eppendorf tubes), storing at -20°C prior to use, and avoiding refreezing and thawing solution because this step can degrade antibiotic especially beta-lactams (Wiegand et al. 2008).

For this study, the MIC determination via broth microdilution was done based on the above recommendations. The antibiotic ampicillin, methicillin and oxacillin were included as positive control to verify the results. Meanwhile, the MIC determination for plant extracts and fractions in this chapter was aided by thiazolyl blue tetrazolium dye (MTT). Presences of viable cells are detected by dehydrogenase enzyme activity of these cells which changed the yellow tetrazole in MTT to insoluble formazon product in purple (Mosmann 1983).

3.1.3 Kinetic growth curve experiment

Kinetic growth curve is a method used to monitor bacterial growth over time. The method is very convenient since growth of the test organism is measured by optical property that is the optical density (OD) of test culture (Koutny and Zaoralkova 2005). A kinetic growth curve experiment is fairly similar to MIC determination. Instead of identifying lowest concentration that inhibits bacterial growth at end point, in this experiment, absorbance reading was taken on an hourly basis. The data obtained is used to plot a kinetic growth curve that shows the bacterial growth phase which essentially allows evaluation of an antimicrobial agent's effect on the bacteria (Breidt et al. 1994).

In the study of potential synergism, kinetic growth curve experiment is useful to monitor combination effect of two agents in affecting bacterial growth. Additional

information such as at what phase of bacterial growth inhibition is observed and duration of antibacterial effects are also obtainable. Earlier studies of synergism have selected this experiment to demonstrated interaction of two agents combined (Muroi and Kubo 1996; Din et al. 2013a). For this study, kinetic growth curve experiment was employed to examine any synergistic effect of plant test agents and ampicillin in combination against MRSA growth.

3.1.4 FIC index interpretation

FIC index interpretation is also known as the checkerboard method. This method is widely used in detecting synergy because it is relatively easy. Results from this assay are observed at end-point which is basically assessing the inhibitory activity of two combined agents (White et al. 1996). MIC value is mostly used in assessing synergy interaction of bacterial study. As such, calculation and interpretation of this index is compromised by MIC value (Berenbaum 1984).

Preparation of bacterial culture in FIC index interpretation is similar as for the kinetic growth curves. A bacterium is grown in media in the presence of two agents. Definition of synergistic effect is interpreted at the lowest concentrations of the agents where no bacterial growth observed after an overnight incubation (Takahashi and Kanno 1984). This information is used to calculate FIC index which can be defined as synergy, antagonism or indifference based on the index.

3.1.5 Western blot

Western blot is a fundamental method for protein detection and at the same time allows quantification of protein expression. There are three basic rudiments in Western blot technique; 1) separation by protein size, 2) transfer to a solid support and 3) identifying target protein by using appropriate primary and secondary antibody. During the initial step, proteins in a mixture are separated by their molecular weight via sodium dodecyl (lauryl) sulphate-polyacrylamide (SDS) gel electrophoresis. After the electrophoresis, the proteins are transferred onto a membrane, stained with dyes to confirm presence of band for each protein and then incubated with specific antibody for the protein of interest. After incubation, the gel is washed to remove the unbound antibody. The bound antibody is detected by developing a film or photo imager and quantified based on its' the thickness (Mahmood and Yang 2012).

The first step in Western blot experiment is protein extraction and it is crucial to establish the extract's concentration to confirm mass of protein loaded into each lane during gel electrophoresis to ensure an equivalent analysis of the samples (Mahmood and Yang 2012). In this study, Pierce 660 nm Reagent assay was used to quantify the sample for construction of protein standard curve. An earlier study has suggested that this reagent is stable at room temperature and recognized to provide reproducible and linear results (Antharavally et al. 2009). As for selection of transfer membrane, nitrocellulose membrane was chosen in this study based on its retention abilities and high affinity for protein and it is fairly easy to be blocked to prevent non-specific antibodies interaction (Riis 2001; Mahmood and Yang 2012). In the blocking step, the protein binding capacity of membrane is often saturated with agents like 5% bovine serum albumin (BSA) or non-fat dry milk to

prevent antibody from binding to the membrane and yield a non-specific signal. Another aspect in Western blot analysis is inclusion housekeeping control to confirm that 1) equal amount of protein sample is loaded in each lane; 2) proteins are transferred with equal efficiency from the gel to the membrane and 3) consistent antibody incubation based on signal detection across the different lanes (Johnson 2012).

In Western blot assay, to recognize the targeted protein, the gels will be first probed with primary antibody. The primary antibody detects the precise sequence of amino acid of the targeted protein and binds to it. A secondary antibody which is often conjugated with enzyme like Horse Radish Peroxide (HRP) then probed to detect the primary antibody. This increases the sensitivity of the assay by amplifying the detection signal (Mahmood and Yang 2012).

3.2 AIMS AND OBJECTIVES

The specific objectives of this chapter were:

1. To evaluate anti-MRSA activities of the extracts and fractions obtained from the plants via determination of MIC.
2. To determine the synergistic effects of combination treatments consisting ampicillin and selected extracts or fractions on MRSA growth.
3. To investigate the effects of the selected combination treatments on PBP2a.

3.3 METHODS

3.3.1 Microorganisms

Methicillin sensitive *S. aureus* ATCC 11632 (MSSA) was grown in tryptic soy broth (TSB) (Hi-Media, India) at 37°C for 24 hours with a shaking mode of 220 rotations per minute (rpm). Aliquot from this suspension was streaked on tryptic soy agar (TSA) (Hi-Media, India) and incubated at 37°C for another 24 hours. Two to four single colonies from the TSA plate was inoculated in 10 ml of Muller Hinton broth (MHB) (Hi-Media, India) and allowed to grow at 37°C until it reached exponential stage (2×10^8 CFU/ml). The suspension then was used for broth microdilution assay (section 3.3.3). MRSA ATCC 43300 was grown similarly except all the media used for its growth was supplemented with 2% NaCl (Merck, Germany) and incubation temperature was 35°C. Bacterial stocks were kept at -80°C in TSB added with 10% (v/v) glycerol (Sigma, USA).

3.3.2 Preparation of test agents

Crude ethyl acetate extract of *A. wilkesiana* (9EA) and *D. grandiflora* (75EA) and their respective fractions were dissolved in DMSO (Sigma, USA) at stock concentration of 100 mg/ml. Further dilution was carried out using media and the final concentration of DMSO in the media did not exceed 1%. DMSO did not exert effect in the testing system as shown in our previous work (Othman et al. 2011a).

Antibiotics for susceptibility testing were prepared at 10 mg/ml in sterile distilled water. Tested antibiotics were ampicilin (Amresco, USA), oxacillin (Discovery Fine Chemicals, UK) and methicillin (Sigma, USA).

3.3.3 Assessment of the antibacterial activity via determination of MIC

Broth microdilution method was used to determine the MIC of plant crude extracts, their respective fractions and antibiotics against MRSA and MSSA. A 96-well plate was used to prepare the antimicrobial agents in serial two-fold dilution. This assay was carried out in triplicates on three separate occasions. Antibiotics were tested with concentration ranging from 0.19 to 100 µg/ml and plant extract samples from 0.09 to 12 mg/ml. Bacterial suspensions were prepared according to methods described in Section 3.3.1. Two hundred micro liters (200 µl) of test agents was added into each well of the first row and followed by a serial two-fold dilution. Then 70 µl of appropriate broth was added into every well. This followed by addition of 30 µl of bacterial suspension into every well (which corresponded to 5×10^5 CFU/ml in a final volume of 200 µl per well) (see Figure 3.1). This experiment was done according to guidelines from CLSI 2007 with recommendations adapted from Cos et al. 2006.

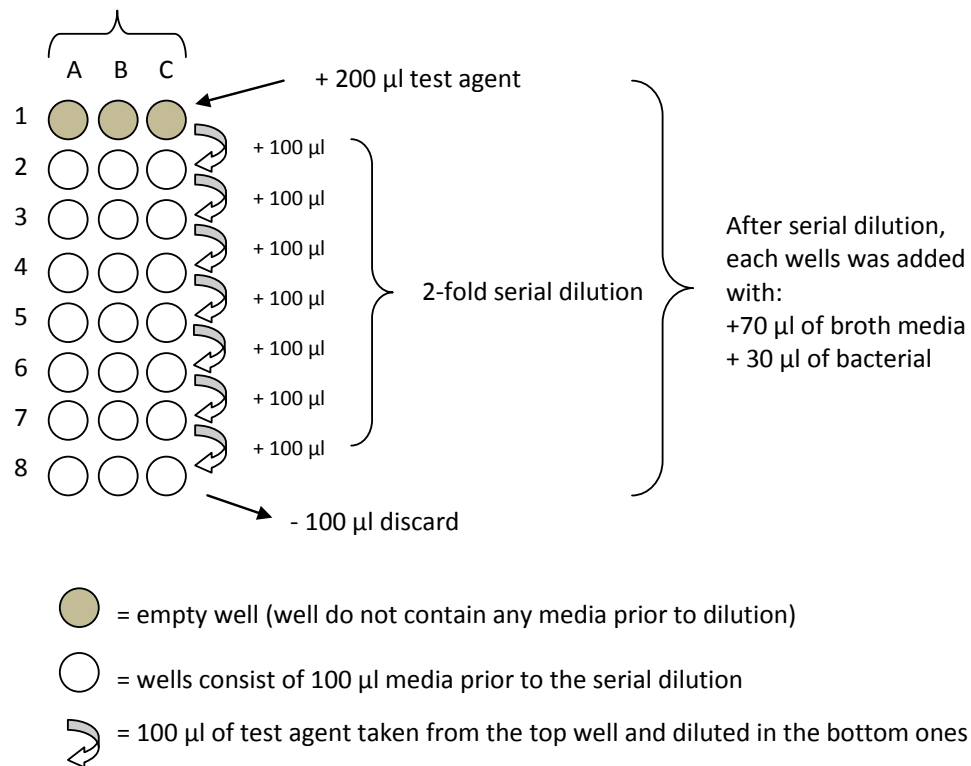


Figure 3.1 Schematic diagram of a set of triplicates used for broth microdilution assay in a 96-well microtiter plate showing serial dilution process and content of each well in the assay.

All experiments in evaluating antimicrobial activities for MRSA were carried out in MH media (+ 2% NaCl) with growth temperature $\leq 35^{\circ}\text{C}$ and results were noted after 24 hours incubation. The susceptible control strain, MSSA ATCC 11632 was grown at 37°C in MH media and incubated for 24 hours. After the incubation period, 20 µl of 5 mg/ml of MTT (Nacalai Tesque, Japan) prepared in sterile distilled water was added to the wells containing plant test agents. The lowest concentration at which color of the broth remained as yellow was identified as the MIC. In the presence of viable cells, the yellow color of MTT will change to purple due to the presence of insoluble formazon.

3.3.4 Kinetic growth curve assay

MRSA was grown in the presence of sub-MIC of ampicillin alone, sub-MIC of plant test agent alone and in combination. Concentrations of test agents used in this experiment were below MIC values to observe effects of these agents on MRSA growth curve. The selection of plant extracts and fractions are described in section 3.1 (see Table 3.1), 3.4.2 and 3.4.3. Tested concentration range for ampicillin was from 25 to 0.78 µg/ml ($1/2 \times \text{MIC}$ to $1/64 \times \text{MIC}$). Plant test agents were tested at $1/4 \times \text{MIC}$, $1/8 \times \text{MIC}$ and $1/16 \times \text{MIC}$. The respective MIC values for ampicillin, plant test agents were obtained from broth microdilution assay as described above

Each well consisting test agent either alone or in combination was inoculated with 30 µl of MRSA suspension that corresponds to 5×10^5 CFU/ml. Following were the different conditions of MRSA growth in details;

- a) MRSA alone; 170 µl of MHB (+ 2% NaCl) and 30 µl of bacterial suspension
- b) MRSA in the presence of ampicillin (concentration range $1/2 \times \text{MIC}$ to $1/64 \times \text{MIC}$); 50 µl of ampicillin solution, 120 µl of MHB (+ 2% NaCl) and 30 µl bacterial suspension
- c) MRSA in the presence of plant test agent (concentration used $1/4 \times \text{MIC}$, $1/8 \times \text{MIC}$, $1/16 \times \text{MIC}$); 50 µl of, plant test agent, 120 µl of MHB (+ 2% NaCl) and 30µl of bacterial suspension
- d) MRSA in combination of ampicillin and plant test agent; 50 µl of plant test agent, 50 µl of ampicillin solution, 70 µl of MHB (+ 2% NaCl) and 30 µl of bacterial suspension

All combinations were tested in triplicates on three different occasions. The assay was carried out using a 96-well microtitre plate. Plate was incubated at 35°C and the OD was recorded at every hour for 24 hours (600 nm wavelength) with a multimode reader (Varioskan Flash, Thermo Scientific, USA).

3.3.5 FIC index interpretation- Checkerboard method

FIC indices for the combination treatments were calculated. Antibiotics were tested with concentration ranging from 0.78 to 25 µg/ml in combination with plant test agents from 0.05 to 3.0 mg/ml. The formula used was:

FIC ampicillin = MIC of ampicillin in combination / MIC ampicillin alone

FIC test sample = MIC of test sample in combination / MIC of test sample alone

FIC index = FIC of ampicillin + FIC of test sample

Definition of the interaction between ampicillin and the test samples based on the FIC index is shown in Table 3.2 (White et al. 1996).

Table 3.2 Interpretation of FIC index based on study by White et al. 1996.

FIC Index	Interpretation
0.5	Synergy
>0.5 but 4.0	Indifference
4	Antagonism

3.3.6 Determination of ampicillin MIC in presence of plant test agents

A broth microdilution method was employed to determine the MIC of ampicillin in presence of plant test agent. The antibiotics and test samples were prepared and combined as described in section 3.3.4. Both antibiotic and plant test agent were combined at concentration lower than MIC. The assay was carried out using a 96-well microtitre plate. Plate was incubated at 35°C for 24 hours.

After the incubation period, 20 µl of 5 mg/ml of MTT (Nacalai Tesque, Japan) prepared in sterile distilled water was added to the wells containing combination of ampicillin with plant test agent. The lowest concentration of ampicillin in the combination treatment at which the color of the broth remained as yellow was identified as the new MIC for ampicillin. All combinations were tested in triplicates on three different occasions.

3.3.7 Western blot analysis

The bioactive fraction FC-B and 75EA-L-F10 was selected for study of the effects of plant test agents in combination with ampicillin on PBP2a expression. The Western blot assay was carried out on the combination treatments i) 1/32 x MIC ampicillin + 1/4 x MIC FC-B and ii) 1/64 x MIC ampicillin + 1/4 x MIC 75EA-L-F10 and on each test agent separately. The tested concentrations for ampicillin were 1/16 x MIC, 1/32 x MIC and 1/64 x MIC. Plant test agents were tested at 1/4 x MIC and 1/8 x MIC. The MIC values for ampicillin and the selected fractions were obtained from broth microdilution assay (see section 3.3.3 and 3.3.6). All the tested concentrations in this analysis are less than MIC value. This is because MIC of the tested antibacterial agents will inhibit MRSA growth therefore, prevents any

observation on how each agent affects the PBP2a expression For rationale of combination treatments selection see section 3.1 and 3.4.7 (Table 3.7).

2.3.7.1 Materials

Tris base (Promega, USA), ethylenediaminetetraacetic acid (EDTA) disodium salt (Dihydrate) (Promega, USA), DNase I (Thermo Scientific, USA), powdered lysozyme (Thermo Scientific, USA), protein inhibitor cocktail (Thermo Scientific, USA), sodium chloride (Sigma, USA), sodium dihydrogen phosphate (Nacalai Tesque, Japan), Urea (Bio-Rad, USA), Pierce 660 nm protein assay kit (Thermo Scientific, USA), NuPAGE® 4-12% Bis-Tris Precast Gels (1.0mm X 12 well) (Invitrogen, USA), NuPAGE® 4-12% Bis-Tris Gel Cassettes (1.0mm X 10 well) (Invitrogen, USA), glycine (Bio-Rad, USA), UltraPure™ sodium dodecyl sulphate (SDS) (Invitrogen, USA), acrylamide (monomer) (Nacalai Tesque, Japan), ammonium persulfate (Bio-Rad, USA), N, N, N', N'-tetramethylethylenediamine (Nacalai Tesque, Japan), BenchMark™ pre-stained protein ladder (Invitrogen, USA), spectra multicolor broad range protein ladder (Thermo Scientific, USA), lithium dodecyl sulphate (LDS) sample buffer- non-reducing (4X) (Thermo Scientific, USA), Coomassie brilliant blue R-250 (Thermo Scientific, USA), glacial acetic acid (Merck, Germany), methanol (Merck, Germany), Tween® 20, Sigma Ultra (Sigma-Aldrich, USA), and TMB membrane peroxidase substrate (1-C) (KPL, UK)

2.3.7.2 Protein extraction

MRSA culture was grown in ampicillin alone, bioactive fractions alone (FC-B and 7EA-L-F10) and in combinations until late exponential phase in a 20 ml centrifuge tube. Size of inoculums used was 5×10^5 CFU/ ml in final volume of 10 ml. All the samples were prepared as described section 3.3.1 and 3.3.2 prior to the experiment.

Bacterial protein was extracted by preparing the lysates in an extraction buffer containing Tris and EDTA. Culture supernatants were harvested via 7,500 rpm centrifugation at 4°C for 10 minutes (Eppendorf Centrifuges 5810, Germany). Collected pellets were then treated with 150 mg/ml lysozyme, DNase and protein inhibitors cocktail and subjected to 2 hours incubation at 37°C (Witeg WiseCube® WIS-20, Germany). The pellets were exposed to 15 minutes sonication (Bandelin Sonorex, Germany) in ice-bucket to enhance cell disruption. Following 15 minutes of centrifugation at 12,000 rpm (Eppendorf Centrifuges 5810, Germany), the pellets were obtained as the insoluble cytoplasmic protein extracts that were harvested in 50 µl elution buffer containing Tris, urea and sodium dihydrogen phosphate. Then, the protein mixture was shaken at 1000 rpm, at 18°C for 30 minutes. This followed by centrifugation for 15 minutes, at 12,000 rpm at 18°C. The supernatant was obtained as protein lysate which its concentrations were measured using Pierce 660 nm reagent assay (Thermo Scientific, USA).

3.3.7.3 SDS-PAGE and Western Blot Assay

Extracted protein (3 µg/ml) was solubilised with 4 X LDS sample buffer (for optimal separation) and subjected to SDS - polyacrylamide (12%) gel electrophoresis run at 120 V. Upon completion the gel was stained in Coomassie Blue (Thermo Scientific, USA) staining solution until a clear background was obtained for scanning with GS-800™ calibrated densitometer (Bio-Rad, USA).

In Western blot analyses, electrophoresed gels were transferred to BioTrace™ NT nitrocellulose transfer membrane (Pall, USA). Membranes were incubated overnight at 4°C in gelatin from cold water fish skin (blocking agent) (Sigma, USA). Gelatin from cold water fish skin was used since it is an effective blocker that do not gel at 4°C, therefore considered best for Western blotting (Riis 2001). The production of PBP2a from MRSA was detected by probing the membranes with mouse anti-PBP2a primary antibody (Denka Seiken, Japan) and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Thermo Scientific, USA) with dilution factor of 1:10,000. The same membranes were re-probed with anti-mouse horseradish peroxidase-linked secondary antibody (Abcam, UK) diluted to 1:10,000 to facilitate colorimetric detection with 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate (Nacalai Tesque, Japan). GAPDH was selected as loading control for Western blot experiments in this study. It is a cytoplasmic enzyme (~37 kDa) involved in bacterial energy generation, a process which is essential for growth (Polgar 1964; Alvarez 2003). The enzyme is extensively preserved across all species, including bacteria, fungi and protozoa (Winram and Lottenbery 1996; Oliveira et al. 2012). Whereas, the antibodies used in this study were also used in preceding work on PBP2a expression; hence verify their reliabilities for this study (Ohwada et al. 1999; Katayama et al. 2003; Dordel et al. 2014).

Assay response was recorded using GS-800™ calibrated densitometer (Bio-Rad, USA) and recorded based on visual band intensity. Densitometry quantification of Western blot images were performed using Image J 1.38 programme (Windows version of National Institute of Health (NIH) Image software) and results were scored in percentage of expression (%) normalized to GAPDH control.

3.4 RESULTS

3.4.1 Minimum inhibitory concentration of antibiotics

Both MRSA and MSSA were tested for antibiotic susceptibility against the following; ampicillin, methicillin and oxacillin. The MIC values were summarized in Table 3.3. As expected, a higher concentration of all the antibiotics tested was required to inhibit the growth of MRSA compared to MSSA. The difference is between 8-fold (ampicillin and methicillin) and 32-fold (oxacillin). The rest of the experiments in this chapter were continued using ampicillin. Ampicillin served as choice of drug for this study because it's a useful drug used for treatment of bacterial infections caused by both Gram positive and negative. The WHO listed ampicillin as one of the most important medication required in basic health care system. It is also co-administered with sulbactam (a drug that inhibits beta-lactamase) for treatment of penicillin-resistant strains (WHO 2013).

Table 3.3 Minimum inhibitory concentration value for MRSA ATCC 43300 and MSSA ATCC 11632 against beta-lactam drugs Values represent triplicates of three independent experiments.

Beta-lactam drugs	MIC ($\mu\text{g/ml}$)	
	MRSA ATCC 43300	MSSA ATCC 11632
Ampicillin	50.00	6.25
Methicillin	20.00	2.50
Oxacillin	10.00	0.31

3.4.2 Minimum inhibitory concentration of crude extract *A. wilkesiana* and fractions

The crude extract *A. wilkesiana* (9EA), 6 major fractions and 13 semi-pure fractions were tested against MRSA and MSSA. Results revealed only one of the major fractions (9EA-FA) did not exhibit any anti-staphylococcal activity. All the other major fractions exhibited antibacterial activity against MRSA and MSSA with the MIC ranging from 1.5 to 12 mg/ml . A lower MIC value was recorded for MSSA in comparison to MRSA for all active major fractions except 9EA-FB. Interestingly, 9EA-FB was identified as the most potent major fraction. The MIC value was 8-fold lower than crude extract, 9EA. (Table 3.4).

The MIC value against MRSA and MSSA for 9EA- FC was 2-fold lower than 9EA. 9EA-FD whereas, has the same MIC value as 9EA for both MRSA and MSSA. 9EA-FC and 9EA-FD were selected for further isolation and purification. This is because the other major fractions were either 1) inactive against MRSA and MSSA, 2) did not demonstrate synergism with ampicillin in combination treatment, 3) insufficient in amount for further fractionation, or 4) highly polar fraction that is unsuitable for fractionation using LC methods employed in this study (Chapter 2).

Evaluation of semi-pure fractions obtained from 9EA-FC and 9EA-FD revealed that 6 out of 13 fractions exhibited anti-MRSA activity. The MIC values however, were not markedly lowered after the purification. The observed decreased in MIC values were between 2 to 4-fold only (Table 3.4). The lowest MIC value against MRSA and MSSA for a semi-pure fraction was 1.5 mg/ml for fraction FC-A. The other semi-pure fractions (that demonstrated activity) have MIC of 3 mg/ml against MRSA and 1.5 mg/ml against MSSA.

For the kinetic growth curve study, the fractions 9EA-FB, 9EA-FC, 9EA-FD, and FC-B were selected. These fractions demonstrated stable activity (consistent activity against MRSA and MSSA each time of the testing), available in sufficient amount for further assays and are mid-polar fractions which means they can be further separated using the LC methods employed in this study.

3.4.3 Minimum inhibitory concentration of crude extracts *D. grandiflora* and fractions

The MIC of *D. grandiflora*'s leaves extract (75EA-L), bark extract (75EA-B) and their respective major fractions were determined. Crude extract 75EA-L and 75EA-B yielded 10 and 9 fractions respectively. Antibacterial effects against MRSA and MSSA for 75EA-L were encouraging with an MIC of 3 mg/ml against both strains (see Table 3.5). However, all major fractions from the leaves extract did not affect MRSA and MSSA survival with exception of 75EA-L-F10 with relatively low MIC of 0.75 mg/ml. This is the major fraction that possessed the most potent anti-staphylococcal activity amongst the entire plant sample tested in this study

MIC for 75EA-B against MRSA and MSSA was 3 mg/ml (Table 3.5). After fractionation, only 6 out of 9 major fractions exhibited antibacterial activity. The MIC of 5 of these major fractions (75EA-B-F5, 75EA-B-F6, 75EA-B-F7, 75EA-B-F8 and 75EA-B-F9) remained at 3 mg/ml for MRSA. Three of these major fractions (75EA-B-F6, 75EA-B-F7 and 75EA-B-F8) also have same MIC (3 mg/ml) for MSSA. The other two major fractions (75EA-B-F5 and 75EA-B-F9) have MIC value of 1.5 mg/ml against MSSA which was 2-fold lower compared to the crude, 75EA-B.

The major fraction 75EA-L-F10 was not further fractionated because the fraction was highly polar, therefore unsuitable to be fractionated via LC methods used in this study. Nevertheless, this fraction was selected for kinetic growth curve study. 75EA-B and major fractions derived from it was excluded from further fractionation and kinetic growth curve study due to poor yield.

Table 3.4 Minimum inhibitory concentration of crude extract *A. wilkesiana* (9EA) and its fractions against MRSA ATCC 43300 and MSSA ATCC 11632. Values represent triplicates of three independent experiments.

Test Agents	MIC (mg/ml)	
	MRSA ATCC 43300	MSSA ATCC 11632
<i>Crude extract</i>		
9EA	12.0	6.0
<i>Major fractions</i>		
9EA-FA	-	-
^a 9EA-FB	1.5	1.5
^{a,b} 9EA-FC	6.0	3.0
^{a,b} 9EA-FD	12.0	6.0
9EA-FE	6.0	1.5
9EA-FF	3.0	1.5
<i>Semi-pure fractions</i>		
FC-A	1.5	1.5
^a FC-B	3.0	3.0
FC-C	3.0	3.0
FC-D	-	-
FC-E	-	-
FC-F	-	-
FC-G	-	-
FD-A	3.0	3.0
FD-B	-	-
FD-C	3.0	-
FD-D	3.0	-
FD-E	-	-
FD-F	-	-

- not active, did not exhibit any inhibitory activity in the assay

^a Fraction selected for kinetic growth curve study

^b Fraction selected for further separation via LC methods (Chapter 2)

Table 3.5 Minimum inhibitory concentration for MRSA ATCC 43300 and MSSA ATCC 11632 against crude extracts of *D. grandiflora* (75EA-L and 75EA-B) and fractions. Values represent triplicates of three independent experiments.

Test Agents	MIC (mg/ml)	
	MRSA ATCC 43300	MSSA ATCC 11632
<i>Crude extracts</i>		
75EA-L	3.0	3.0
75EA-B	3.0	3.0
<i>Major Fractions (75EA-L)</i>		
75EA-L-F1	-	-
75EA-L-F2	-	-
75EA-L-F3	-	-
75EA-L-F4	-	-
75EA-L-F5	-	-
75EA-L-F6	-	-
75EA-L-F7	-	-
75EA-L-F8	-	-
75EA-L-F9	-	-
^a 75EA-L-F10	0.75	0.75
<i>Major Fractions (75EA-B)</i>		
75EA-B-F1	-	-
75EA-B-F2	-	-
75EA-B-F3	-	-
75EA-B-F4	1.5	3.0
75EA-B-F5	3.0	1.5
75EA-B-F6	3.0	3.0
75EA-B-F7	3.0	3.0
75EA-B-F8	3.0	3.0
75EA-B-F9	3.0	1.5

- not active, did not exhibit any inhibitory activity in the assay

^a Fraction selected for kinetic growth curve study

3.4.4 Kinetic growth curves- effects of *A. wilkesiana* and ampicillin

Combination treatments of ampicillin and plant samples from *A. wilkesiana* generally exhibited a larger inhibitory effect on the growth of MRSA compared to a single agent treatment. A maximum inhibition of up to 80% was observed when MRSA was treated with crude extract *A. wilkesiana* (9EA) alone indicating their antibacterial effects (see Figure 3.2).

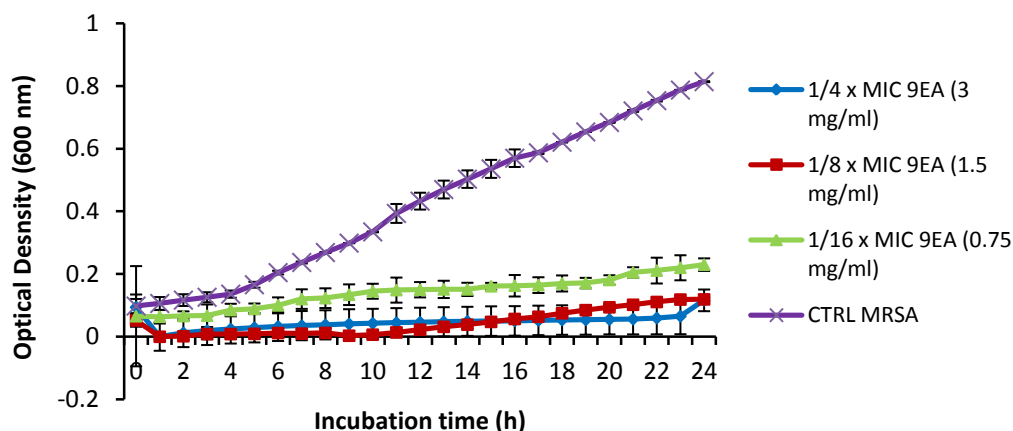


Figure 3.2 Effects of *A. wilkesiana* crude extract (9EA) on MRSA ATCC 43300 growth survival curve over 24 hours. The concentrations used were below the agents MIC (i.e. 1/4 x MIC, 1/8 x MIC and 1/16 x MIC). Cell growth was measured by using OD at 600 nm at indicated time point. The curves represent triplicates of three independent experiments. Error bars show the standard deviation (AMP= ampicillin, MIC= minimum inhibitory concentrations).

Ampicillin when tested at concentrations ranging from 1/2 x MIC (25 µg/ml) to 1/64 x MIC (0.78 µg/ml), demonstrated a concentration dependant antimicrobial activity on MRSA. Treatment with ampicillin alone (1/32 and 1/64 x MIC) depicted an exponential growth similar to control MRSA (Figure 3.3).

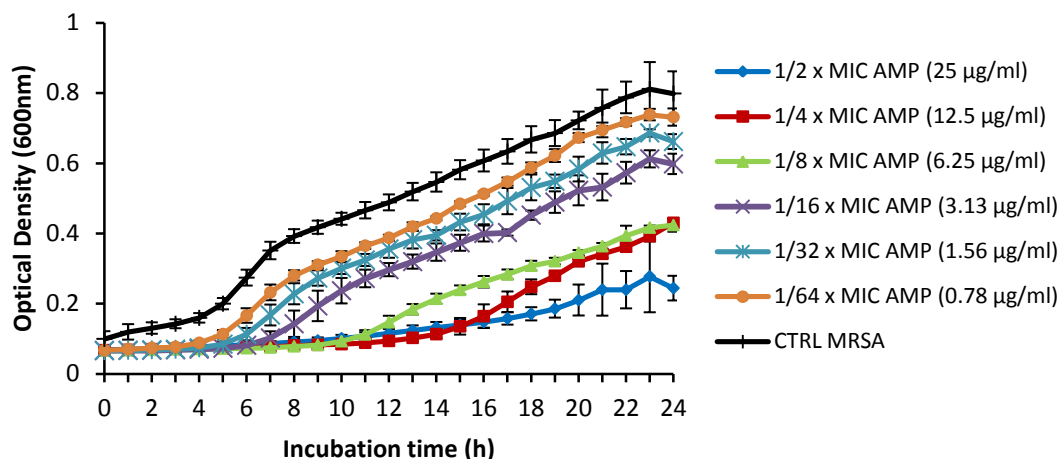


Figure 3.3 Effects of ampicillin on MRSA ATCC 43300 growth survival curve over 24 hours. The concentrations used were below the agents MIC (i.e. 1/64 x MIC to 1/2 x MIC). Cell growth was measured by using OD at 600 nm at indicated time point. The curves represent triplicates of three independent experiments. Error bars show the standard deviation (AMP= ampicillin, MIC= minimum inhibitory concentrations).

3.4.4.1 Effects of crude extract 9EA and ampicillin

Combination treatment of 9EA and ampicillin showed total suppression of the bacterial survival (see Figure 3.4). The OD of MRSA growth started declining from the 5th hour and continued to decrease to a point below zero suggesting bacterial cells lysis. Treatment with 9EA alone appeared to have rapid antibacterial action based on sharp decline of OD within 2 hours of incubation. However, after 12 hours MRSA seemed to have a slow and steady increase in the cell number. In contrast, the combination treatment indicated potent anti-MRSA activity.

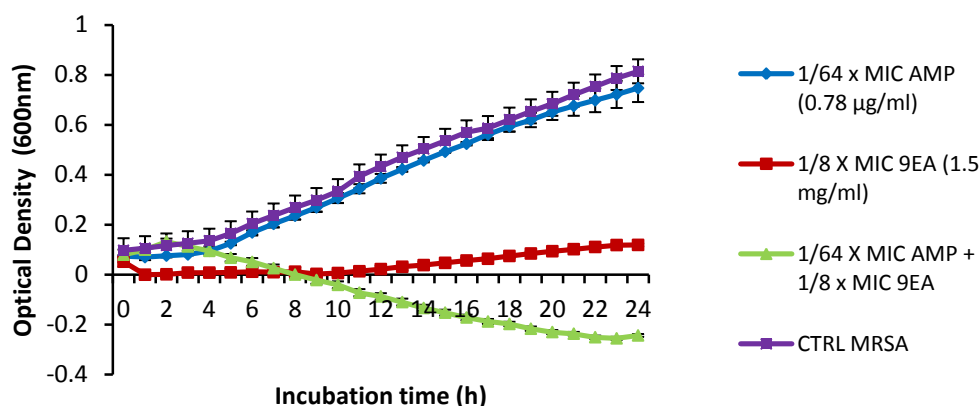


Figure 3.4 Effects of ampicillin alone, 9EA alone and in combination on MRSA ATCC 43300 growth survival curve over 24 hours. The concentrations used were below the agents MIC (i.e. 1/64 x MIC AMP and 1/8 x MIC 9EA). Cell growth was measured by using OD at 600 nm at indicated time point. The curves represent triplicates of three independent experiments. Error bars show the standard deviation (AMP= ampicillin, MIC= minimum inhibitory concentrations).

3.4.4.2 Effects of bioactive fraction 9EA-FB and ampicillin

Fraction 9EA-FB exhibited anti-MRSA action as shown in the Figure 3.5. Fraction 9EA-FB did not demonstrate any additive inhibitory effect on MRSA when combined with ampicillin. When 9EA-FB was introduced as treatment at different concentrations (1/2 to 1/16 x MIC), MRSA growth were seen to be affected adversely in concentration dependant manner (Figure 3.5a). At 1/16 x MIC 9EA-FB, there was no obvious antibacterial effect and the growth curve is similar to control MRSA. Experiment conducted on combination of 9EA-FB and ampicillin (Figure 3.5b) revealed that the combination treatment had poorer anti-MRSA activity compared to treatment with 9EA-FB alone which suggested presence of antagonistic effects in presence of ampicillin. The examination on MRSA growth curves in 9EA-FB (1/2 to 1/8 x MIC) treated cultures suggested that the fraction may have affected the lag phase in which this phase was extended up to 7 hours compared to control MRSA that progressed to grow into exponential phase by the 4th hour. Besides that, the growth curves of 9EA-FB

treated cultures also demonstrated shorter exponential with slow growth as a result of 9EA-FB antimicrobial action.

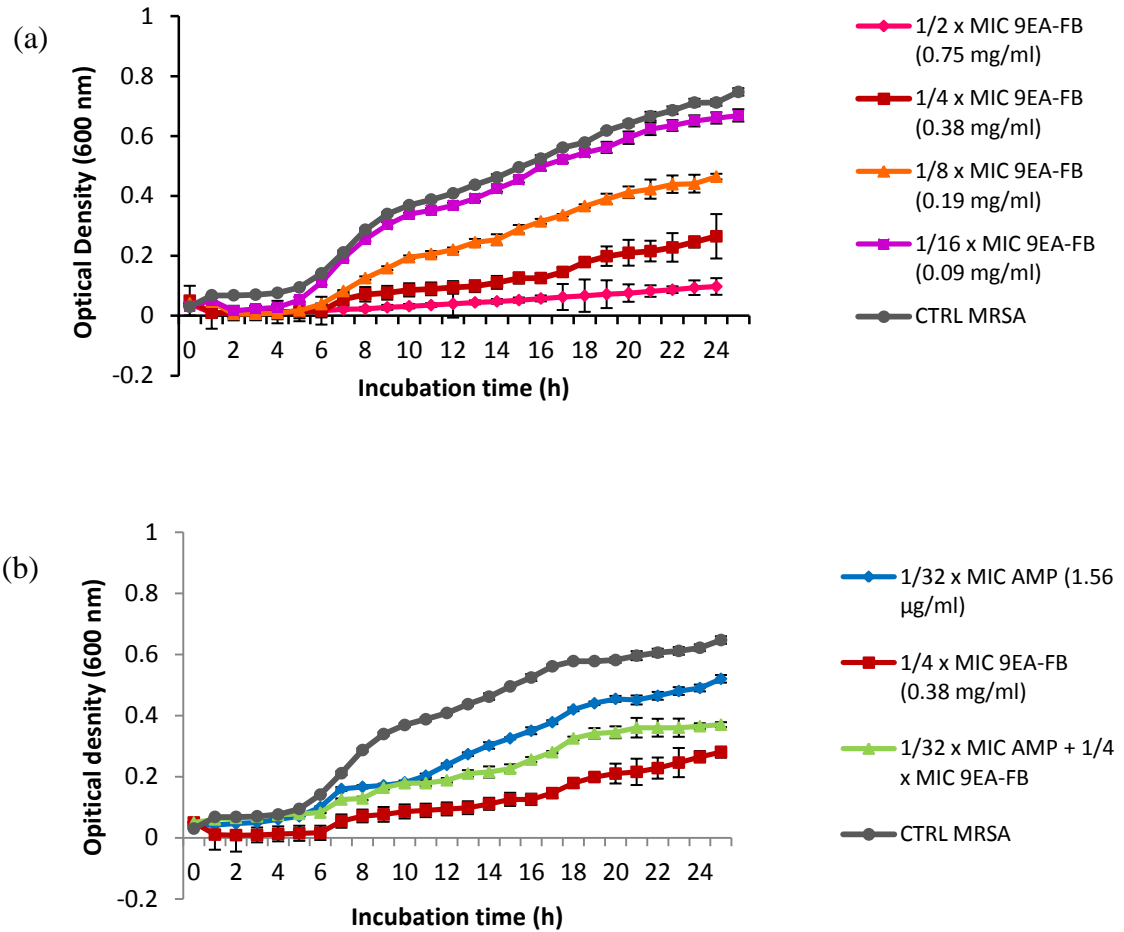


Figure 3.5

Effects of a) 9EA-FB alone and b) 9EA-FB in combination with ampicillin alone on MRSA ATCC 43300 growth survival curve over 24 hours. The concentrations used were below the agents MIC (i.e. 1/32 x MIC AMP and 1/16 x MIC to 1/2 x MIC 9EA-FB). Cell growth was measured by using OD at 600 nm at indicated time point. The curves represent triplicates of three independent experiments. Error bars show the standard deviation (AMP= ampicillin, MIC= minimum inhibitory concentrations).

3.4.4.3 Effects of bioactive fraction 9EA-FC and ampicillin

Fraction 9EA-FC was experimented in growth curve assay at $1/4 \times \text{MIC}$, $1/8 \times \text{MIC}$ and $1/16 \times \text{MIC}$. The concentration $1/4 \times \text{MIC}$ showed the most potent inhibitory activity on MRSA upon combination with ampicillin and $1/16 \times \text{MIC}$ were devoid of any effect. Hence, we chose to present combination treatment of $1/4 \times \text{MIC}$ 9EA-FC and ampicillin in this section.

Figure 3.6 represent MRSA growth in combination treatment of 9EA-FC with ampicillin. The graph revealed that combination of ampicillin and 9EA-FC managed to reduce MRSA growth curve to a below zero point with no increase detected in the OD during the incubation time compared to treatment of ampicillin or 9EA-FC alone. This indicated that no growth occurred during incubation and possible cells lysis occurring at 19th hour. Also evident was the introduced treatments ($1/32 \times \text{ampicillin}$ - blue line, $1/4 \times \text{MIC}$ 9EA-FC- red line and combination of both- green line) have similar growth rate during the first four hours of incubation. Nevertheless, as the bacterial cultures continued to grow into exponential stage (around 5th hour), suppression of cell division took place in the cultures that were exposed to 9EA-FC. This observation enable us to deduce that, besides attenuating MRSA growth throughout the exponential stage, 9EA-FC also appeared to act more actively in inhibiting MRSA growth at starting point of stationary phase (18th hour) based on the trend of declining OD.

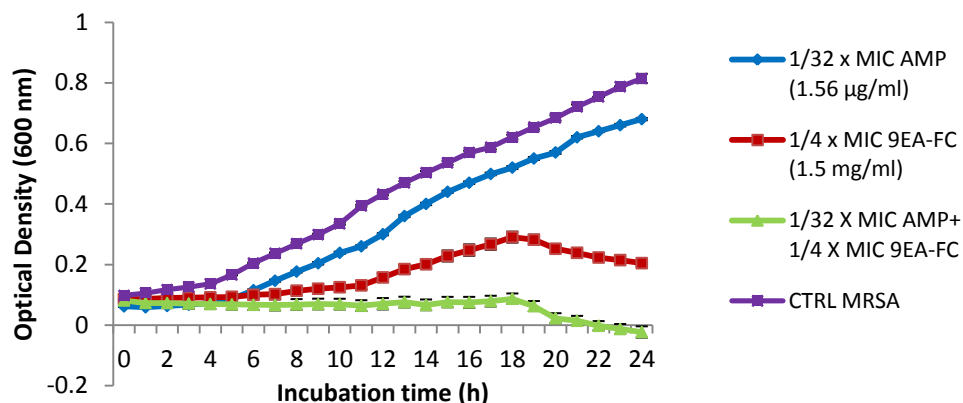


Figure 3.6 Effects of ampicillin alone, 9EA-FC alone and in combination on MRSA ATCC 43300 growth survival curve over 24 hours. The concentrations used were below the agents MIC (i.e. 1/32 x MIC AMP and 1/4 x MIC 9EA-FC). Cell growth was measured by using OD at 600 nm at indicated time point. The curves represent triplicates of three independent experiments. Error bars show the standard deviation (AMP= ampicillin, MIC= minimum inhibitory concentrations).

3.4.4.4 Effects of bioactive fraction 9EA-FD and ampicillin

Fraction 9EA-FD was studied at 1/4 x MIC, 1/8 x MIC and 1/16 x MIC. The concentration 1/4 x MIC demonstrated the most potent suppression on MRSA growth when combined with ampicillin (Figure 3.7). Combination treatment has displayed marked inhibition on MRSA growth during the entire incubation time. Examination of these growth curves showed that during the first 4 hours, similar OD was recorded for cultures from all three treatments. However OD of cultures exposed to ampicillin (blue line) started to increase at 5th hour showing similar trend as observed for control untreated. The increasing trend for these two growth curves (blue and purple line) at 5th was showing that the bacterium was progressing to grow into exponential phase. Whereas, the lower OD recorded from 5th hour

onwards in MRSA cultures treated with 9EA-FD alone showed that inhibitory activity by this fraction potentially took place during the bacterium's entry to exponential phase.

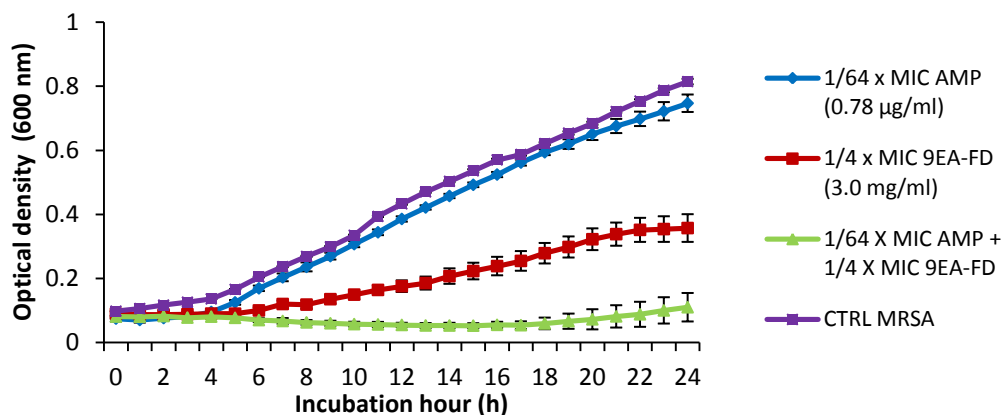


Figure 3.7 Effects of ampicillin alone, 9EA-FD alone and in combination on MRSA ATCC 43300 growth survival curve over 24 hours. The concentrations used were below the agents MIC (i.e. 1/64 x MIC AMP and 1/4 x MIC 9EA-FD). Cell growth was measured by using OD at 600 nm at indicated time point. The curves represent triplicates of three independent experiments. Error bars show the standard deviation (AMP= ampicillin, MIC= minimum inhibitory concentrations).

3.4.4.5 Effects of bioactive fraction FC-B and ampicillin

Fraction FC-B was tested at 1/4 x MIC, 1/8 x MIC and 1/16 x MIC. The concentration 1/4 x MIC suppressed MRSA growth the most when combined with ampicillin and presented here in Figure 3.8.

MRSA growth in all treatments appeared similar for the first 3 hours of incubation. As the incubation time continues, growth curves of cultures exposed to FC-B (red and green lines) were substantially lower compared to cultures grown in ampicillin alone. This suggests that the antibacterial action possibly took place at 4th hour of incubation which is at the beginning of exponential phase. The OD of MRSA in FC-B alone although approximately

2-fold lower in contrast to control MRSA and ampicillin, the growth curve indicated that bacteria continues to grow. Whilst, in combination treatment, the OD had a very minor increase till the end of incubation period.

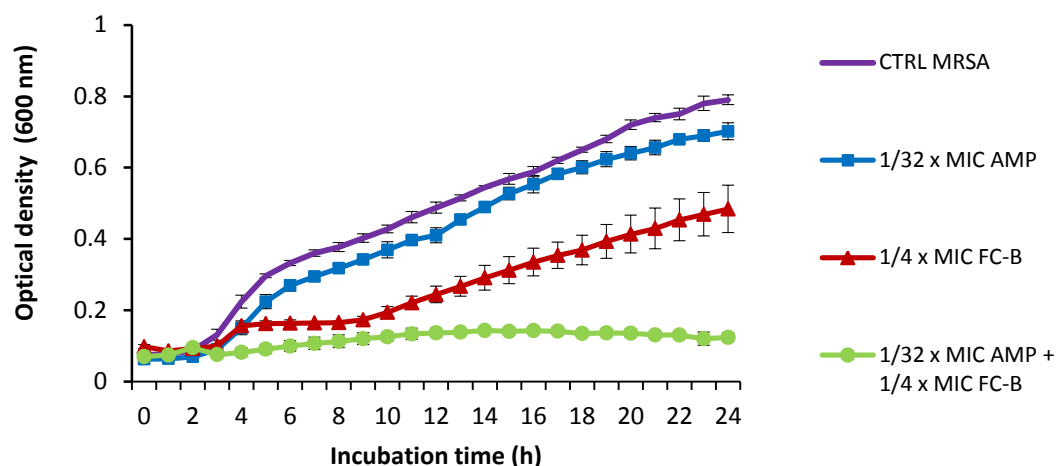


Figure 3.8 Effects of ampicillin alone, FC-B alone and in combination on MRSA ATCC 43300 growth survival curve over 24 hours. The concentrations used were below the agents MIC (i.e. 1/32 x MIC AMP and 1/4 x MIC FC-B). Cell growth was measured by using OD at 600 nm at indicated time point. The curves represent triplicates of three independent experiments. Error bars show the standard deviation (AMP= ampicillin, MIC= minimum inhibitory concentrations).

3.4.5 Kinetic growth curves – effects of *D. grandiflora* and ampicillin

Combination treatments of ampicillin and plant samples from *D. grandiflora* resulted in strong inhibition on MRSA growth with limited growth throughout incubation period. MRSA when grown in presence of ampicillin alone showed a concentration dependant inhibitory activity (see section 3.4.4).

When tested alone, the crude extract *D. grandiflora* (75EA-L) demonstrated suppression on MRSA growth approximately 40% at 1/4 x MIC (Figure 3.9). At 1/8 x MIC (red line), 75EA-L displayed certain level of inhibition on MRSA growth with the OD was seen increasing steadily but still lower compared to control MRSA. Whereas, 75EA-L at 1/16

x MIC (green line) showed a weak antibacterial activity based on growth curve that was similar to control MRSA till 15th hour of incubation.

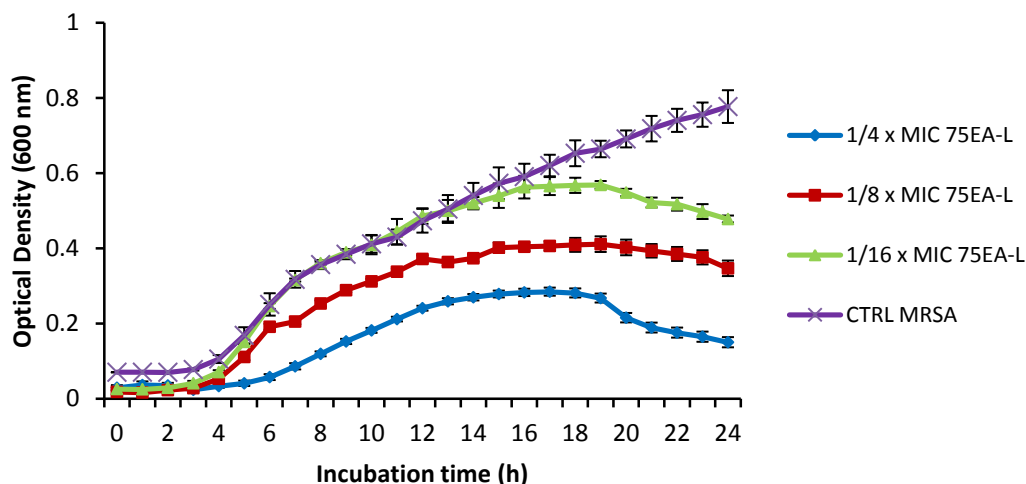


Figure 3.9 Effects of *D. grandiflora* crude extract (75EA-L) on MRSA ATCC 43300 growth survival curve over 24 hours. The concentration used were below the agents MIC (i.e. 1/4 x MIC, 1/8 x MIC and 1/16 x MIC). Cell growth was measured by using OD at 600 nm at indicated time point. The curves represent triplicates of three independent experiments. Error bars show the standard deviation (AMP= ampicillin, MIC= minimum inhibitory concentrations).

3.4.5.1 Effects of crude extract 75EA-L with ampicillin

Figure 3.10 is showing the effects of 75EA-L and ampicillin on MRSA growth. Combination treatment of 75EA-L with ampicillin was able to completely inhibit MRSA growth as no increase in OD recorded during incubation time. In fact, a very strong antibacterial effect was exhibited in the combination treatment. Although the OD of culture grown with 75EA-L alone (red line) increased steadily till the 18th hour before declining, the growth was still 2-fold lower compared to cultures grown in 1/64 x MIC ampicillin alone (blue line) and control MRSA (purple line). Inhibition of growth possibly began during lag phase and continued through the exponential stage based OD that increased slowly from 0 to 18th hour

in treatment of 75EA-L alone while in untreated cultures, increase in OD was noted from the 4th hour onwards with a clearly defined exponential stage.

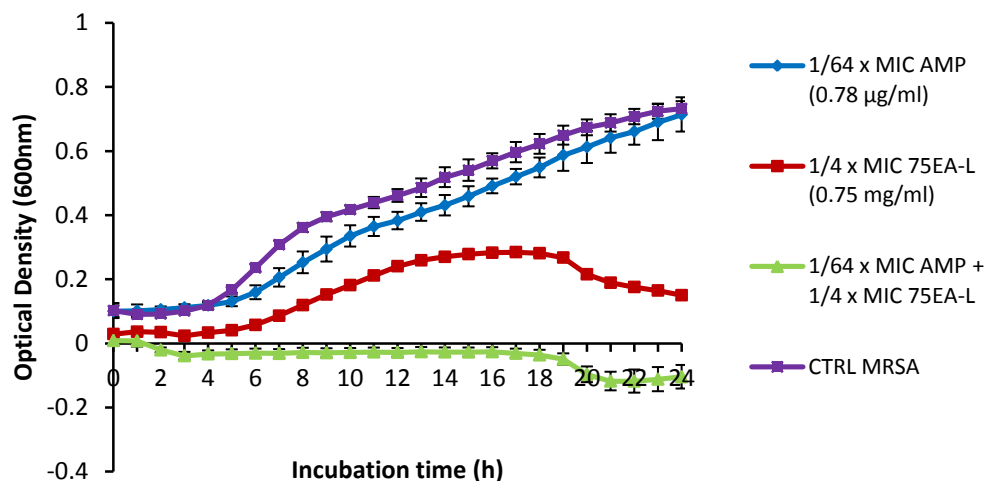


Figure 3.10 Effects of ampicillin alone, 75EA-L alone and in combination on MRSA ATCC 43300 growth survival curve over 24 hours. The concentrations used were below the agents MIC (i.e. 1/64 x MIC AMP and 1/4 x MIC 75EA-L). Cell growth was measured by using OD at 600 nm at indicated time point. The curves represent triplicates of three independent experiments. Error bars show the standard deviation (AMP= ampicillin, MIC= minimum inhibitory concentrations).

3.4.5.2 Effects of bioactive fraction 75EA-L-F10 with ampicillin

Fraction 75EA-L-F10 in combination with ampicillin successfully prevented MRSA growth and remained constant from the start of the experiment until the end of incubation time (Figure 3.11). In presence of 75EA-L-F10 (red line), the growth of MRSA was suppressed by about 2-fold. It was evident that MRSA was suppressed at lag phase which possibly prevented the bacterium from growing into exponential phase. This can be visualized in the graph between the 5th to 7th hour, in which the curve of control MRSA increased rapidly, but growth curves of 75EA-L-F10 treated cultures were demonstrating almost a still OD.

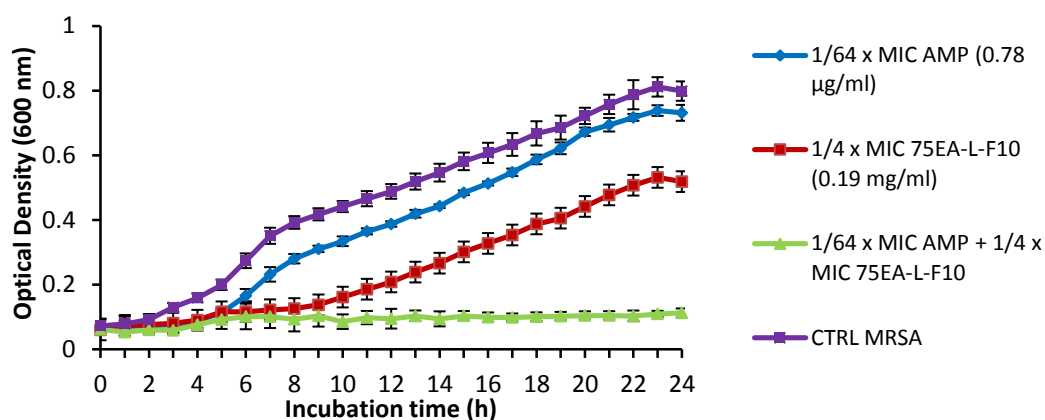


Figure 3.11 Effects of ampicillin alone, 75EA-L-F10 alone and in combination on MRSA ATCC 43300 growth survival curve over 24 hours. The concentrations used were below the agents MIC (i.e. 1/64 x MIC AMP and 1/4 x MIC 75EA-L-F10). Cell growth was measured by using OD at 600 nm at indicated time point. The curves represent triplicates of three independent experiments. Error bars show the standard deviation (AMP= ampicillin, MIC= minimum inhibitory concentrations).

3.4.6 FIC index interpretation for combination treatments

Interpretation of FIC index was carried out for combination treatments consisting of ampicillin and plant test agents. In total, 108 combinations were tested. The FIC index for each combination is summarized in Table 3.6.

Forty-eight combinations showed synergistic based on $FIC < 0.5$. Out of these, 27 combinations were with plant test agents at 1/4 x MIC. A drop in the number of synergistic interaction recorded with a lower concentration of these plant test agents. There were only 17 and 4 synergistic combination recorded for 1/8 x MIC and 1/16 x MIC of the plant test agents respectively. Similar observation was recorded for ampicillin where the highest number of synergistic interaction was recorded in combination treatment containing 1/4 x

MIC ampicillin. At lower concentration of ampicillin, there is increasingly more combination treatment exhibiting no antibacterial effect. Interestingly, presence of $1/2 \times \text{MIC}$ ampicillin in combination treatments produced almost all indifference and two treatments without any antibacterial effect.

Further examination on the FIC indices indicated that the synergy interactions in inhibiting MRSA growth depends on concentration of both of the agents as lesser combinations with FIC index < 0.5 (synergy) was observed when concentration of the test agents were reduced. When both of the agents were present at the lowest tested concentration ($1/16 \times \text{MIC}$ plant test agents and $1/64 \times \text{MIC}$ ampicillin), no synergistic interaction or antimicrobial action was observed. At $1/8 \times \text{MIC}$, only crude extract 9EA was seen to interact synergistically with $1/64 \times \text{MIC}$ ampicillin.

Each plant test agent was compared individually, and crude extract *A. wilkesiana* (9EA) interacted synergistically with ampicillin in 11 synergistic combinations. This was followed by crude extract *D. grandiflora* (7EA-L) and its bioactive fraction, 75EA-L-F10 with 9 combinations each. Fraction FC-B, 9EA-FC and 9EA-FD recorded 8, 6 and 5 combinations with synergistic interaction respectively. Combination of $1/8 \times \text{MIC}$ 9EA + $1/64 \times \text{MIC}$ ampicillin was identified as the combination expressing the highest synergistic interaction.

Table 3.6 Fractional inhibitory concentration indices interpretation for combinations of sub-MIC ampicillin with sub-MIC crude extract and bioactive fractions from *A. wilkesiana* and *D. grandiflora*. Experiments were carried out in triplicates on three separate occasions (MIC= minimum inhibitory concentrations).

Sub-MIC extract/fraction (mg/ml)	FIC Index (Interpretation)					
	Combined with Sub-MIC ampicillin (µg/ml)					
	1/2 x MIC (25.00)	1/4 x MIC (12.50)	1/8 x MIC (6.25)	1/16 x MIC (3.13)	1/32 x MIC (1.56)	1/64 x MIC (0.78)
9EA						
1/4 x MIC (3.00)	0.75 (I)	0.50 (S)	0.38 (S)	0.31 (S)	0.28 (S)	0.27 (S)
1/8 x MIC (1.50)	0.63(I)	0.38 (S)	0.25 (S)	0.19 (S)	0.16 (S)	0.14 (S)
1/16 x MIC (0.75)	0.56(I)	0.31(S)	-	-	-	-
9EA-FC						
1/4 x MIC (1.50)	0.63(I)	0.38 (S)	0.25 (S)	0.19 (S)	0.16 (S)	-
1/8 x MIC (0.75)	0.57(I)	0.31 (S)	0.19 (S)	-	-	-
1/16 x MIC (0.38)	-	-	-	-	-	-
9EA-FD						
1/4 x MIC (3.00)	0.75(I)	0.50 (S)	0.38 (S)	0.31 (S)	0.28 (S)	-
1/8 x MIC (1.50)	0.63(I)	0.38 (S)	-	-	-	-
1/16 x MIC (0.75)	-	-	-	-	-	-
FC-B						
1/4 x MIC (0.75)	0.75(I)	0.43 (S)	0.38 (S)	0.31 (S)	0.28 (S)	-
1/8 x MIC (0.38)	0.65(I)	0.36 (S)	0.25 (S)	0.19 (S)	-	-
1/16 x MIC (0.19)	0.56(I)	0.31 (S)	-	-	-	-
75EA-L						
1/4 x MIC (0.75)	0.75 (I)	0.50 (S)	0.38(S)	0.31(S)	0.28(S)	0.27(S)
1/8 x MIC (0.38)	0.63(I)	0.38(S)	0.25(S)	0.19(S)	-	-
1/16 x MIC (0.19)	0.56(I)	0.31(S)	-	-	-	-
75EA-L-F10						
1/4 x MIC (0.19)	0.75(I)	0.50(S)	0.38(S)	0.31(S)	0.28(S)	0.27(S)
1/8 x MIC (0.09)	0.63(I)	0.38(S)	0.25(S)	0.19(S)	-	-
1/16 x MIC (0.05)	0.53(I)	0.28(S)	-	-	-	-

I= abbreviation for indifference

S= abbreviation for synergy

- = no effect was observed/ no antimicrobial activity

For interpretation of FIC index; synergism 0.5, indifference >0.5 but 4.0, and antagonism 4 (White et al. 1996).

3.4.7 Summary of synergistic interaction for combination treatment with ampicillin and plant test agents

The two methods applied in studying synergism between plants test agents and ampicillin confirmed that the combination treatments can potentiate the antibacterial effects against MRSA. Growth curve experiments presented possible synergistic interaction visually whilst the FIC index gave a definite quantitative measure of the synergism present. Based on these two experiments, a reduced MIC of ampicillin against MRSA was determined in presence of these plant test agents (see Table 3.7).

Interestingly, all the major fractions (9EA-FC and 9EA-FD) and semi-pure fraction (FC-B) appeared to have a lower ability to reduce the MIC value of ampicillin compared to the crude extract *A. wilkesiana* (9EA). There was only 32-fold reduction compared to 64-fold reduction of MIC value of ampicillin respectively (see Table 3.7). However, the concentration of FC-B needed to suppress MRSA in combination treatment is 0.75 mg/ml is 2-fold lower compared to 9EA. This can be readily justified based on the fact that FC-B is a semi-pure fraction, hence, the active metabolites in this fraction is concentrated. Both plant test agents from *D. grandiflora* exhibited similar ability to reverse MRSA sensitivity to ampicillin by 64-fold. Our results showed that we could reverse the resistance of MRSA to ampicillin via combination treatment. Based on the results from these two experiments we selected two combination treatments for the study of PBP2a expression in MRSA. The two combinations are $1/32 \times \text{MIC ampicillin} + 1/4 \times \text{MIC FC-B}$ and $1/64 \times \text{MIC ampicillin} + 1/4 \times \text{MIC 75EA-L-F10}$ which lowered MIC of ampicillin to 1.56 $\mu\text{g/ml}$ and 0.78 $\mu\text{g/ml}$.

Table 3.7 Minimum inhibitory concentration ampicillin in combination treatment of extracts and fractions from *A. wilkesiana* and *D. grandiflora*. Experiments were carried out in triplicates on three separate occasions (MIC= minimum inhibitory concentrations).

Combined with (sub-MIC= mg/ml)	New MIC ampicillin (µg/ml)
Ampicillin alone	50.00
+ 9EA (1/8 x MIC =1.50)	0.78
+ 9EA-FC (1/4 x MIC = 1.50)	1.56
+ 9EA-FD (1/4 x MIC = 3.00)	1.56
+ FC-B (1/4 x MIC = 0.75)	1.56
+ 75EA-L (1/4 x MIC =0.75)	0.78
+ 75EA-L-F10 (1/4 x MIC = 0.19)	0.78

3.4.8 Inhibition of PBP2a by FC-B

PBP2a band was absent in MSSA strain which was used as control in our study, thus confirming that this strain is methicillin susceptible and do not produce the resistant protein, PBP2a. GAPDH which is the housekeeping protein was expressed in all the tested samples

Ampicillin was tested at two different concentrations; 1/16 x MIC (3.13 µg/ml) and 1/32 x MIC (1.56 µg/ml), and from Figure 3.12a (lane 3 and 4), it is evident that the antibiotic intensified production of PBP2a in MRSA as represented by the very intense bands. The PBP2a band's intensity in treatment of ampicillin alone was stronger compared to control MRSA suggesting induction of PBP2a production in presence of ampicillin. Densitometry analysis showed that the expressed bands were 124.02% and 148.10% for 1/16 x MIC ampicillin and 1/32 x MIC ampicillin respectively, compared to control MRSA.

Based on Figure 3.12a (lane 5 and 6), FC-B at $1/4 \times \text{MIC}$ (0.75 mg/ml) and $1/8 \times \text{MIC}$ (0.38 mg/ml) have adverse effect on PBP2a production. No band was observed at $1/4 \times \text{MIC}$ of FC-B. Weak band intensity was recorded at $1/8 \times \text{MIC}$ FC-B and densitometry analysis showed only 6.15% of PBP2a expression (Figure 3.12b). In the combination treatment ($1/4 \times \text{MIC}$ FC-B + $1/32 \times \text{MIC}$ ampicillin - Figure 3.12a, lane 7) no PBP2a band was found.

3.4.9 Inhibition PBP2a by 75EA-L-F10

The results of the treatments consisting ampicillin alone, 75EA-L-F10 alone and in combination is depicted in Figure 3.13. As expected the MSSA strain did not express any PBP2a band indicating absence of the protein. Bands for the housekeeping protein, GAPDH were expressed in all treated cultures.

MRSA cultures in ampicillin alone ($1/32 \times \text{MIC} = 1.56 \mu\text{g/ml}$ and $1/64 \times \text{MIC} = 0.78 \mu\text{g/ml}$), expressed a very intense PBP2a bands (Figure 3.13a, lane 3 and 4). These bands were expressed as high as 157.82% in treatment of $1/64 \times \text{MIC}$ ampicillin in contrast to control MRSA when quantified using the densitometric analysis. Therefore suggest that presence of ampicillin induces production of PBP2a.

Based on the Western blot image (Figure 3.13a, lane 5 and 6), 75EA-L-F10 has suppressed production of PBP2a at $1/4 \times \text{MIC}$ (0.19 mg/ml) and $1/8 \times \text{MIC}$ (0.09 mg/ml) in MRSA. Faint PBP2a bands were observed at $1/4 \times \text{MIC}$ and $1/8 \times \text{MIC}$ of 75EA-L-F10. Densitometry analyses showed their expression were 17.35% and 18.30% respectively (Figure 3.13b). No PBP2a band was detected in combination treatment of $1/64 \times \text{MIC}$ ampicillin + $1/4 \times \text{MIC}$ 75EA-L-F10 (Figure 3.13a, lane 7).

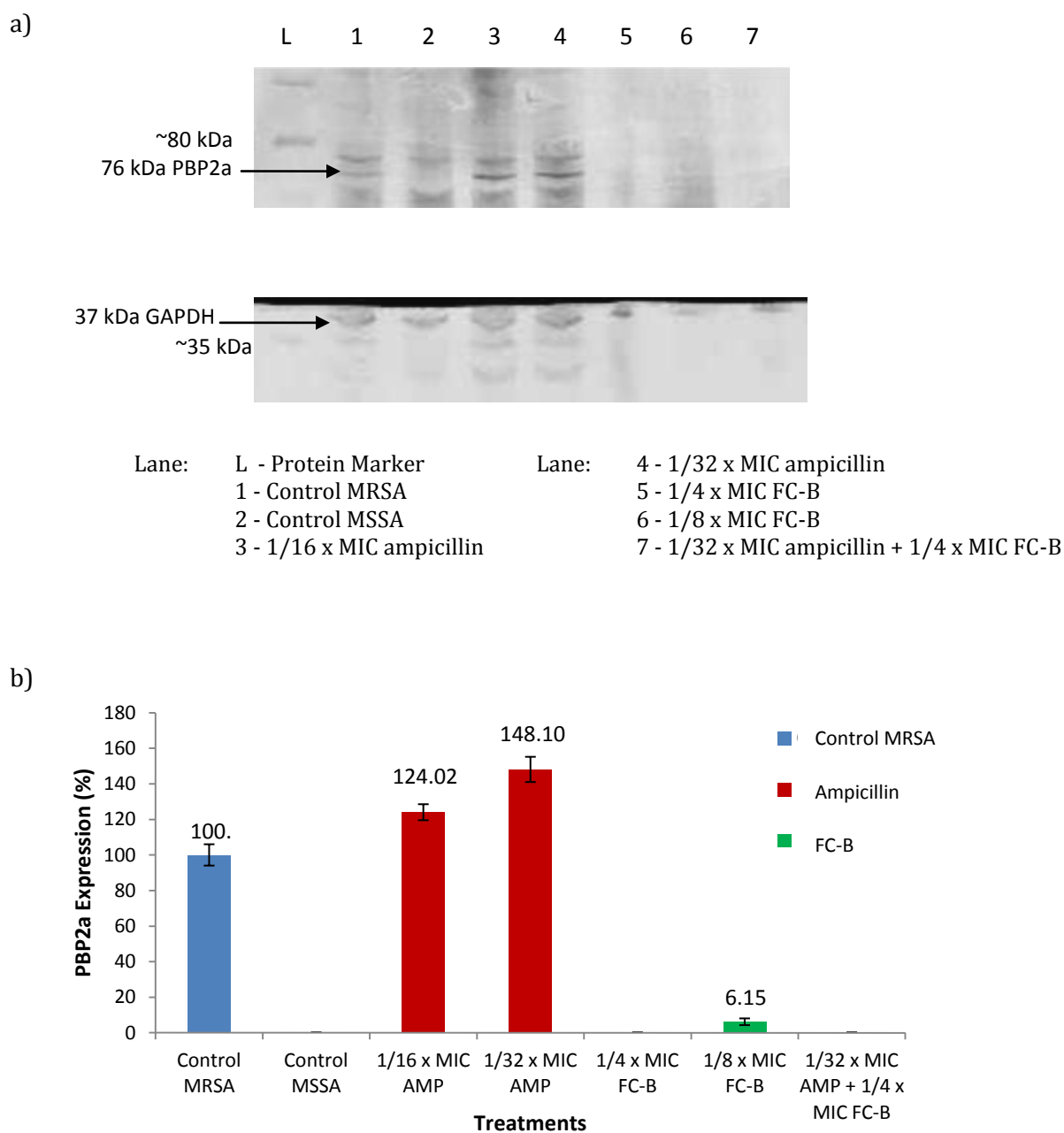


Figure 3.12 a) Western blot of PBP2a expression in MRSA cells treated with FC-B and ampicillin. The extracted protein (3 μ g/ml) was subjected to 12% SDS-PAGE prior to the blotting and presence of PBP2a was detected with mouse anti-PBP2a primary antibody and anti-mouse horseradish peroxidase-linked secondary antibody. b) Quantitative densitometric analysis of PBP2a expression in MRSA. PBP2a bands expression were normalized to GAPDH control (AMP= ampicillin, MIC= minimum inhibitory concentration, n=2).

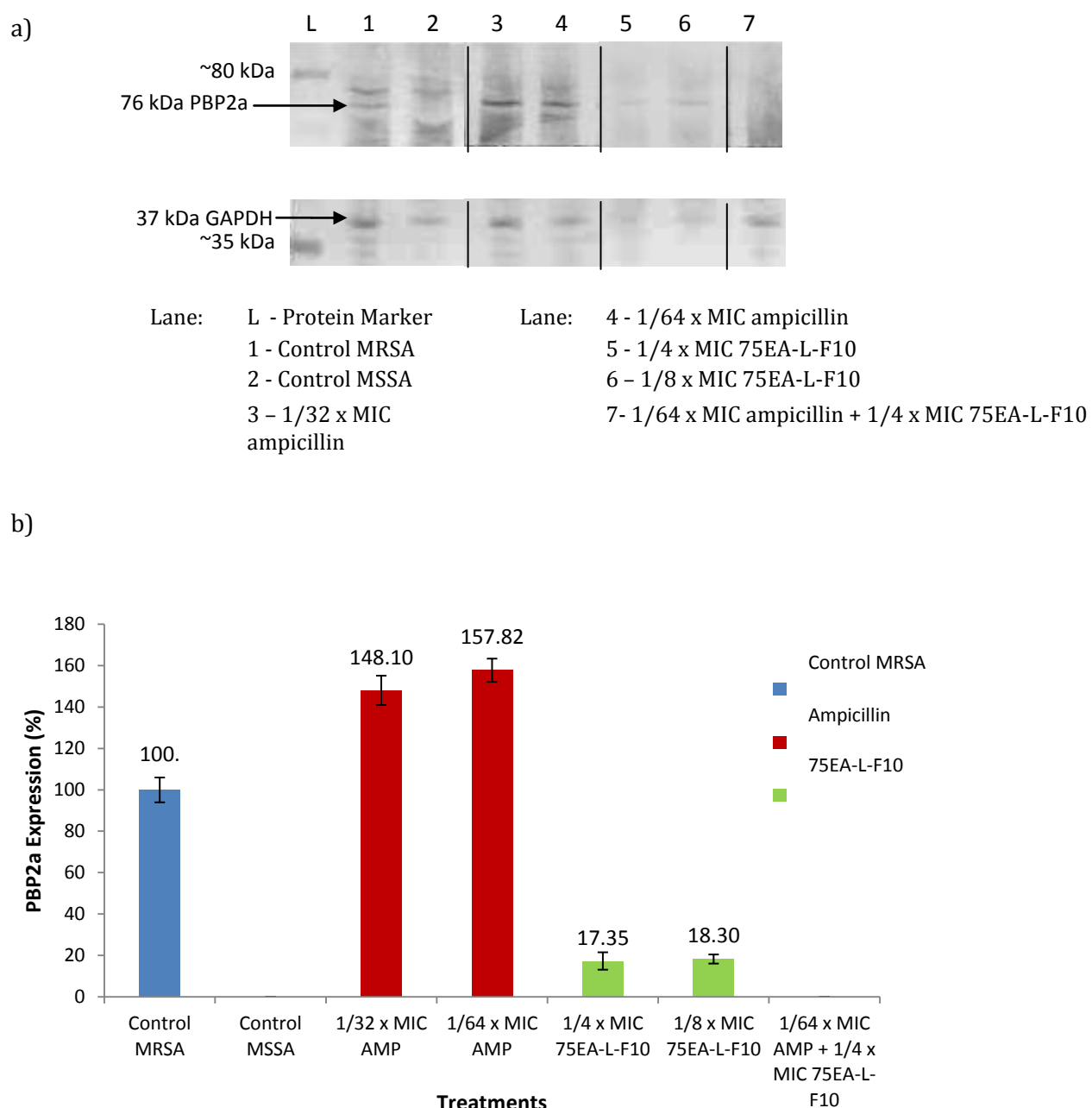


Figure 3.13

a) Western blot of PBP2a expression in MRSA cells treated with 75EA-L-F10 and ampicillin. The extracted protein (3 µg/ml) was subjected to 12% SDS-PAGE prior to the blotting and presence of PBP2a was detected with mouse anti-PBP2a primary antibody and anti-mouse horseradish peroxidase-linked secondary antibody. b) Quantitative densitometric analysis of PBP2a expression in MRSA. PBP2a bands expression were normalized to GAPDH control (AMP= ampicillin, MIC= minimum inhibitory concentration, n=2).

3.5 DISCUSSION

Experiments conducted in this chapter aimed to 1) evaluate antibacterial effects of selected crude extracts and fractions from *A. wilkesiana* and *D. grandiflora*, 2) investigate their possible synergistic action with a beta-lactam antibiotic, ampicillin as antibacterial agent and 3) study the effect of selected synergistic combination treatments on PBP2a expression. Assessment of the MIC values revealed that the selected plant test agents demonstrated anti-MRSA and anti-MSSA activities. Study of combination treatment has shown that synergistic interaction occurred between the plant test agents and ampicillin in suppressing MRSA growth. We also observed that we can reverse MRSA resistant to ampicillin by using combinational therapy with plant metabolites. Study on PBP2a expression indicated that the combination treatments suppressed the production of this protein.

3.5.1 Evaluation of Antimicrobial Activity

Susceptibility testing was employed initially to confirm resistance of MRSA strain used in this study. The MIC of methicillin against MRSA (strain ATCC 43300) value was 20 µg/ml confirmed that this strain is resistant based on breakpoints interpretations; of susceptible ≤ 4 µg/ml and resistance ≥ 8 µg/ml (Brown 2001). Resistant to methicillin also means resistant to all other beta-lactams and cephalosporins (CLSI 2007). We also observed that this strain (MRSA ATCC 43300) showed high resistance (MIC = ≥ 10 µg/ml) to ampicillin and oxacillin. The MIC of ampicillin against MSSA ATCC 11632 strain was 6.25 µg/ml, which is within the acceptable range of susceptibility. A beta-lactam antibiotic inhibits the PBPs involved in late stage of peptidoglycan biosynthesis. Interference with peptidoglycan biosynthesis causes deformities in the bacterial cell wall and eventually leads to cell death due to high internal osmotic pressure (Lambert 2004).

Nevertheless, there are at least two mechanisms that Staphylococci can evade beta-lactam toxicity which is by production of PBP2a and expression of β -lactamases (Gillespie et al. 1985). Due to expression of these two proteins, MRSA confers resistance to beta-lactams which was evidently shown by the high MIC values recorded for these antibiotics in this study. To counteract the resistant mechanism displayed by MRSA, we tested crude extracts and fractions from *A. wilkesiana* and *D. grandiflora* as potential anti-MRSA agents.

Evaluation of crude extract of *A. wilkesiana* (9EA) showed that the extract has modest anti-MRSA activity (MIC = 12 mg/ml). When the extract was fractionated, the obtained major fractions and semi-pure fractions demonstrated a better antibacterial action based on a decrease of the MIC ranging from 1.5 to 6.0 mg/ml. This showed an increase of up to 8-fold for anti-MRSA activity among the bioactive fractions. 9EA demonstrated anti-MSSA activity with MIC of 6 mg/ml. The major fractions and semi-pure fractions exhibited a better activity as represented by MIC values between 1.5 to 3.0 mg/ml. Based on these MIC values, it is likely that 9EA and its fractions contained bioactive plant metabolites that contributed to the observed antibacterial activities.

The anti-staphylococcal activities shown by 9EA and its respective fractions is in agreement with previous studies on isolation of antibacterial compounds such as corilagin, ethyl gallate and geraniin from *A. wilkesiana* extracts (Adesina et al. 2000; Shimizu et al. 2001; Sato et al. 2004). Investigation on mode of action has shown that these compounds cause cell lysis leading to bacterial death in *S. aureus* species (Din et al. 2013a). This indicates that the similar phenomena could have contributed to anti-MRSA and anti-MSSA activity of plant test agents obtained from *A. wilkesiana*. Besides, the compounds corilagin and geraniin were found to infer with the mechanism of resistance displayed by MRSA namely, production of PBP2a (Shimizu et al. 2001). In a separate

study, tannins (which are frequently isolated from *A. wilkesiana*) have been reported to be capable of binding to peptidoglycan and destroy the bacterial cell wall integrity which eventually results in growth inhibition (Zhao et al. 2001; Zhao et al. 2002). The information compiled from previous studies shows *A. wilkesiana* may affect *S. aureus* growth in several ways. Therefore, offer a plausible explanation for the plant test agents' activities against both MRSA and MSSA in this study.

MIC evaluation of *D. grandiflora* extracts (75EA-L and 75EA-B) indicated that these extracts are more active in inhibiting MRSA growth compared to 9EA based on the MIC value of 3 mg/ml. This value is 4-fold lower compared to MIC of 9EA against MRSA, which was 12 mg/ml. Despite of the relatively better antibacterial activity, only 7 out of 19 isolated major fractions from the *D. grandiflora* extracts affected MRSA growth. There was only one active fraction (75EA-L-F10) derived from 75EA-L and 6 active fractions from 75EA-B. These major fractions displayed minimal improvement in the antibacterial effect after the fractionation process. The crude extracts and the major fractions exhibited similar antibacterial effects on MSSA. MIC of crude extracts against MSSA is 3 mg/ml. This value remained the same in some of the major fractions. Whilst in others the MIC value decreased by just 2-fold except in 75EA-L-F10 in which we observed a 4-fold decrease.

The inhibitory activity shown by both the crude and fractions from *D. grandiflora* in this experiment suggested prospective existence of antimicrobial properties since earlier research conducted in our lab has shown that these extracts have anti-staphylococcal activities and contain biologically active phytochemicals such as tannins, alkaloids, flavonoids, and steroids (Othman et al. 2011a, Othman et al. 2011b). Literature search indicated limited studies on antibacterial agents of *D. grandiflora* while most of the isolated active compounds reported to have anti proliferative actions against

cancer cells (Tsukiyama et al. 2010; Kaweetripob et al. 2012). Hence, while we are able to deduce that the antibacterial effects exhibited by *D. grandiflora* are contributed by the plant metabolites, we are unable to postulate any mechanism involved in plant's antibacterial activity at this stage due to limited research on the plant.

Evaluation of MIC on crude extracts and fractions obtained from *A. wilkesiana* and *D. grandiflora* has enabled us to identify the fractions that exhibited antibacterial activity. However, we observed that the bioassay-guided fractionation process has resulted in the loss of activity in some of the major fractions when they were further separated into semi-pure fractions. Some fractions simply retained their activity after the fractionation process when we hypothesized an improvement in the bioactivity as this process was supposed to concentrate the active metabolites. Our observation suggests that the active metabolites may be spread across several fractions from the extract. The possible reason is that less pure fractions contain a number of compounds that work synergistically to produce the observed antibacterial effect. Fractionation has in fact, "diluted" or "spread" out these compounds (Bucar et al. 2013). Hence, the fraction loses or shows no improvement in its antimicrobial activity when it is separated into smaller units. These reasons explain why a major fraction from 9EA (9EA-FD) exhibited similar MIC value as the crude extract and that all the fractions from 75EA-L lost their antibacterial effect. In the plant kingdom, it is known that the secondary metabolites produced typically have weak antibacterial activity. Nonetheless, since plants adopt strategy of synergism, the produced metabolites interact with each other synergistically in overcoming infections (Hemaiswarya et al. 2008). Hence, this phenomenon may clarify the lost or no change in bioactivity of some fractions.

3.5.2 Combination of crude extracts or bioactive fractions from *A. wilkesiana* and *D. grandiflora* and ampicillin synergistically inhibits MRSA growth

Concept of synergism is applied to treat infections caused by deleterious pathogens to tackle the complex multi-drug resistance issues (Hemaiswarya et al. 2008). A review by Gibbons, compiled antibacterial and modifying resistance properties of compounds from plant origin against Staphylococcal species showing use of plant products as anti-staphylococcal agents (Gibbons 2004). Several compounds, such as epicatechin gallate, totarol and corilagin reversed methicillin-resistance in MRSA by reducing MIC of beta-lactams when combined (Nicolson et al. 1999; Hamilton-Miller and Shah 2000; Shimizu et al. 2001). These findings impetus the hypotheses that usage of plant extracts, especially of ethnobotanical value, in combination with beta-lactam could be a rational strategy in manipulating resistance factor of MRSA, subsequently inhibit MRSA growth.

Results obtained from kinetic growth curve assay and FIC index interpretations have demonstrated that the crude extracts and bioactive fractions from the medicinal plants, *A. wilkesiana* and *D. grandiflora* work synergistically with ampicillin in suppressing MRSA growth. This was evidently depicted in MRSA growth curves in which growth suppression was observed in cultures that were grown in combination treatment. Subsequently, the FIC index interpretation further affirmed the synergistic action. The results from these two experiments also indicated the reversal of ampicillin-resistance in these cultures (MIC of ampicillin changed from 50 µg/ml to 1.56 µg/ml and 0.78 µg/ml) in the presence of plant test agent. Therefore, proposing that presence of these extracts or fractions potentiate antimicrobial action of ampicillin against MRSA.

3.5.2.1 Kinetic growth assay for plant test agent in combination with ampicillin

Kinetic growth assay provided a platform to observe “visually” antibacterial action of all different treatments against MRSA growth curve. All the test agents were tested at concentration below MIC to ensure the concentration used is not affecting cell survival. From our findings, we deduced two prominent patterns of growth curves in combination treatments of ampicillin and plant test agents; 1) growth curves that were reduced with time and reached OD below zero and 2) growth curves that indicated very minimal increase in OD and remained constant during the incubation period.

Generally, the results showed an improved suppression on MRSA growth when the plant test agent and ampicillin were combined. Our findings also revealed that ampicillin when tested alone at $1/32 \times \text{MIC}$ and $1/64$ demonstrated a very poor inhibition of MRSA growth. In fact, the growth curve in these treatments resembles control MRSA. However, the same concentration of ampicillin when combined with plant test agents exhibited marked suppression on MRSA growth. Therefore, we propose that presence of plant test agent potentiates antibacterial effects of ampicillin.

a) *A. wilkesiana* extract and fractions

Examination of MRSA growth curve in presence of 9EA (crude extract *A. wilkesiana*) alone showed a strong antibacterial effects (~80% inhibition). Whilst growth curves of MRSA treated with fractions from 9EA (9EA-FC, 9EA-FD and FC-B) alone revealed that these fractions were less effective (~30 to 40% inhibition) in suppressing MRSA growth compared to 9EA. Nevertheless, both 9EA and these fractions demonstrated an enhanced inhibition on MRSA growth when combined with ampicillin. This shows that 9EA and the fractions possess antibacterial properties that contributed to the observed activities either alone or in combination treatment. These antibacterial properties may have several mechanism of action which affected MRSA growth.

Previous studies have shown that metabolites from *A. wilkesiana* affected growth of *S. aureus* by causing bacterial cell lysis and interfering with bacterial protein production (section 2.5.1). While this offer plausible explanation for suppression of MRSA growth by the plant agents alone, the enhanced anti-MRSA in the combination treatment indicates the plant test agents may target resistance factors in MRSA. Interference with the resistance factor makes the bacterium susceptible to ampicillin, hence, resulting in suppressed MRSA growth curve.

In combination treatment of 9EA and ampicillin, MRSA survival curve was observed to plunge to a point below zero by the 4th hour. The plunging trend in the combination treatment and the strong inhibition by 9EA alone suggests a rapid killing of bacterium possible through lytic action. This is because an aggressive lytic action will disrupt the cell wall and following this event cells are being fragmented and become debris (Jackson and Kropp 1992; Leekha et al. 2011). As such, OD of the bacterial culture may reduce to a point below zero. Extract of *A. wilkesiana* has been previously

recognized to contain an active ingredients ellagitannin that was shown to act synergistically with ampicillin in suppressing *S. aureus* growth. The same research also reported that upon exposure to extract containing ellagitannin, the bacterial cells were observed to have deformed cell surface in addition to indentation which compromised the cell wall's integrity (Din et al. 2013a). How ellagatannins work synergistically with ampicillin is not clear, but the research suggested the synergistic action resulted in *S. aureus* cell lysis. From these reports, we deduced that similar antibacterial action took place in combination treatment leading to total bacterial cell lyses. However, the enhanced antibacterial action can also be an outcome from synergistic interactions such as 1) multi-target effects that cooperate in agonistic way due to the mixture of plant metabolites in 9EA or 2) adverse interaction with resistance mechanism, since our study was carried out on MRSA (Wagner and Ulrich-Merzenich 2009).

An improved antibacterial activity was observed when *A. wilkesiana* fractions (9EA-FC, 9EA-FD and FC-B) were combined with ampicillin. Fraction 9EA-FB although did not exhibit any synergism in inhibiting MRSA growth when combined with ampicillin, the fraction still demonstrated anti-MRSA activity alone. The MRSA growth curve remained almost constant during the incubation period with no or very minimal increase in OD. This indicated the prevention of bacterial growth and reproduction. Exhibition of such effect suggest a potential bacteriostatic action in the combination treatments. Bacteriostatic action is when an agent limits the bacterial growth via interference with bacterial protein production, DNA replication and other aspects of bacterial cellular metabolism (Pankey and Sabath 2004; Leekha et al. 2011). Since PBP2a is a bacterial protein, it is possible that the suggested bacteriostatic action occurred as result of inhibition of this protein. In addition, PBP2a is a resistant protein and interference of its production by the plant test agent could lead to synergistic action as observed in the combination treatments. We postulate this event reversed methicillin

resistance in MRSA and cause the bacterium to be susceptible to ampicillin again in combination treatments. This is further supported by findings of the compounds corilagin and gallic acid in *A. wilkesiana* extracts (Adesina et al. 2000; Madziga HA et al. 2010; Din et al. 2013b). These compounds in a separate study were found to reverse methicillin-resistance in MRSA via PBP2a inhibition (Shimizu et al. 2001).

b) *D. grandiflora* extract and fractions

Investigation on the crude extract of *D. grandiflora* (75EA-L) and a bioactive fraction isolated from it (75EA-L-F10) revealed that both of these plant test agents have similar antibacterial effect on MRSA growth (~40% inhibition) when tested alone. Combination of these plant test agents and ampicillin showed an increased suppression on MRSA growth. The combination of crude extract 75EA-L and ampicillin was observed to exhibit greater inhibition on MRSA compared to combination of 75EA-L-F10 and ampicillin. This is because presence of 75EA-L in the combination treatment was observed to reduce the OD of the bacterial growth to below zero. Whereas 75EA-L-F10 and ampicillin although did not reduce the bacterial growth to below zero, the combination still effectively suppressed MRSA growth since the OD recorded remained almost constant with minor increase .

Growth curve of MRSA in combination treatments consisting plant test agents from *D. grandiflora* displayed a minor or no increase in OD during the incubation period. This observation is identical to growth curve of MRSA in presence of 9EA and ampicillin which represent a bacteriostatic action to a certain extent. The similar MRSA growth curves pattern in presence of 75EA and 75EA-L-F10 implies that same plant metabolites may occur in these test samples and are involved in inhibiting MRSA growth. So far, no antibacterial compound has been isolated from *D. grandiflora*. However, Othman et al.

(2011a and 2011b) detected alkaloids, tannins, saponin and flavonoids in the *D. grandiflora* extracts that showed antibacterial activity.

Plant metabolite such as flavonoid is often attributed to biological activities. Earlier, the compound licoricidin from flavonoid group has been reported to work synergistically with oxacillin and the combination was found to have bacteriostatic action on MRSA. . Mechanism of action of licoricidin is attributed to the compound's affinity to bind to bacterial cell membrane. Since licoricidin targets bacterial cell membrane, the compound was suspected to alter PBP2a's activity in cell wall production indirectly (Hatano et al. 2005). Hence the finding of flavonoid in *D. grandiflora* offers some rationale for the observed anti-MRSA activity of the test agents in growth curve study both alone and in combination. Besides that, previous studies show plant metabolites have tendency to bind protein including PBPs or cell-wall building blocks which explains their antibacterial activity against MRSA (Hatano et al. 2005; Hu et al. 2001; Hu et al. 2002; Shiota et al. 2004). Taken this information together, we hypothesize that *D. grandiflora* test agents contains metabolites which influence production of PBP2a in MRSA which potentiates ampicillin's action in combination treatments. Nevertheless, the occurrence of mixture of plant metabolites in *D. grandiflora* suggests several mechanisms may be involved in its antibacterial activity.

3.5.2.2 Transpiration point of inhibition

Besides giving indication of the type of antibacterial activity, kinetics growth curves can be used to determine the transpiration point of inhibition especially in closed liquid batch culture as implemented in this study. Bacterial reproduction and growth kinetics is divided into four phases; lag or adaptive phase, exponential phase, stationary phase, and death phase (Figure 3.14).

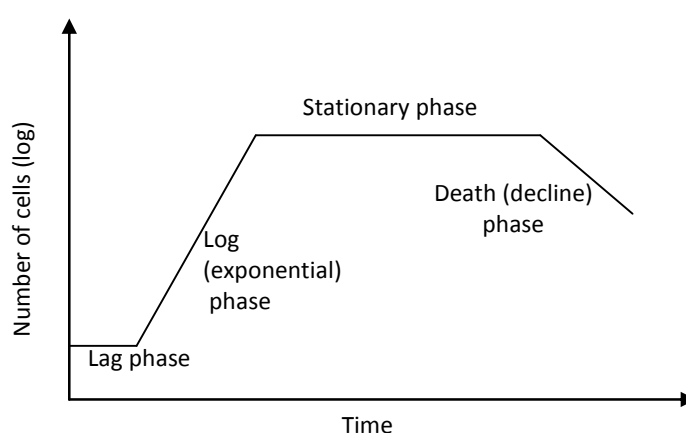


Figure 3.14 A schematic diagram of different stages of bacterial growth in a liquid culture.

An antimicrobial agent's action at a specific phase of bacterial growth provides insight of its mechanism since different metabolic and physiological processes occur during each of these phases. Our findings from MRSA growth curves experiment revealed that inhibition occurred mainly at the growth phase as depicted in Table 3.8.

Table 3.8 Effects of extracts or fractions from *A. wilkesiana* and *D. grandiflora* on MRSA growth phase based on results from kinetic growth curve assays.

Extracts/ fractions	Affected growth phase
9EA	Exponential
9EA-FC	Exponential
9EA-FD	Exponential
FC-B	Exponential
9EA-FB	Lag phase and exponential phase
75EA-L	No clear indication
75EA-L-F10	Lag phase and exponential phase

More than half of our plant test agents affect the exponential growth phase i.e. 9EA, 9EA-FC, 9EA-FD, FC-B, 9EA-FB and 75EA-L-F10 (Table 3.8). Kinetic growth curve experiments results showed that the plant test agents treated cultures experienced a shorter exponential phase with a lower growth compared to control and ampicillin treated cultures. Moreover, in many of the combination treatments, the curves were flat which suggests that the bacteria division was completely suppressed. This indicates probable interference in cell division which involves multiple rounds of DNA synthesis that is controlled by variety gene regulators (Navarro et al. 2010; Rolfe et al. 2012). Besides that, in the case of 9EA-FB and 75EA-L-F10, an extended lag phase was detected for MRSA treated in the respective fraction alone. Lag phase is a particular stage when bacteria equilibrate to adapt to the new environment by undergoing macromolecular repair and synthesis of cellular growth through DNA replications (Dukan and Nyström 1998). Hence, we deduced that a lengthy lag phase in culture treated with these fractions is due to inhibition of DNA replications that delays the cellular growth process. This observation also is reminiscent of the action of fluroquinolones that caused

inhibition of DNA replication in MRSA, leading to a longer lag phase (Venezia et al. 2001).

3.5.2.3 FIC index interpretations for plant test agent in combination with ampicillin

Results of FIC index interpretation can be summarized into 48 combinations of synergistic effects, 16 combinations of indifference interaction (zero-interaction) and 44 combinations that did not exhibit any antibacterial activity. The results also showed that presence of extract or bioactive fraction from *A. wilkesiana* and *D. grandiflora* reduced the MIC of ampicillin to as low as 0.78 µg/ml (64-fold decrease) from the initial value of 50 µg/ml. These findings bear similarities to previous research that used FIC index interpretation method to report increased susceptibility of MRSA to beta-lactam drugs upon combination with active compounds from medicinal plants (Shiota et al. 2004; Hatano et al. 2005).

A smaller value of FIC index indicates better synergism (White et. al. 1996) and analyses of indices showed the index's range was from 0.14 to 0.50 for combinations with synergistic effect. A study of interpretation of checkerboard experiment using FIC index concluded synergism as 4-fold decrease in MIC with an FIC index of ≤ 0.5 and marked synergism as 8-fold decrease in MIC with an FIC index of ≤ 0.25 (Cappelletty and Rybak 1996). Based on these definitions, we were able to demonstrate that 32 of the tested combinations exhibited marked synergism with a decrease in MIC ampicillin ranging from 8 to 64-fold, attesting credibility of the synergistic activity observed.

At the same time, it was observed that, all combinations that consist $1/2 \times \text{MIC}$ ampicillin or $1/2 \times \text{MIC}$ plant test agents produced an index > 0.5 but < 4.0 indicating

indifference activity. The term indifference for interaction between two drugs defined as combined activity equals to the most active agent used alone (Singh et al. 2000). Hence, inhibitory activity observed can be principally attributed to one of the agents in the combination.

3.5.3 Interference of bioactive fractions FC-B and 75EA-L-F10 with PBP2a

Data from Western blot experiment show that production of PBP2a was completely inhibited in combination treatments ($1/32 \times \text{MIC}$ ampicillin + $1/4 \times \text{MIC}$ FC-B and $1/64 \times \text{MIC}$ ampicillin + $1/4 \times \text{MIC}$ 75EA-L-F10). The bioactive fraction FC-B was also found to totally suppress production of PBP2a without being combined with ampicillin. Hence, FC-B alone may serve as a good candidate for anti-MRSA agent. Both FC-B and 75EA-L-F10 when were tested at $1/8 \times \text{MIC}$ indicated attenuation of PBP2a production in MRSA. Based on these results, we deduced that inhibition of PBP2a is one of the mechanisms involved in antimicrobial activity demonstrated by the bioactive fractions in combination treatments in which we observed restoration of ampicillin's activity. Evidence pointing out to PBP2a inhibition suggests that the inhibitory mechanism may have occurred as a result of interference with the expression of *mecA* gene encoding for PBP2a or a direct degradation of PBP2a by the fractions.

The *mecA* gene transcription is regulated by *mecR1-mecI-mecR2* system, a unique three-component arrangement, consisting of a transcriptional repressor (*mecI*), a sensor-inducer (*mecR1*) and an anti-repressor (*mecR2*) (Archer and Bosilevac 2001; Zhang et al. 2001; Arède et al. 2012). Presence of beta-lactam antibiotics has shown to trigger expression of PBP2a in MRSA (Derensinski, 2005). In fact, this is confirmed in our observation where a higher expression of PBP2a was found in MRSA cultures grown in ampicillin. MRSA when exposed to beta-lactams; binding of the antibiotic molecules to

MecR1 domain (sensor-inducer receptor) induce expression of *mecA* and *mecR1-mecI-mecR2* genes (see Figure 3.15). Induction of *mecA* by MecR1 was identified weak and extremely slow (Ryffell et al. 1992; Hackbarth and Chambers 1993). This inefficiency however is compensated by presence of MecR2, an anti-repressor which inactivates the function of MecI (repressor) by destabilizing the binding of this repressor to *mecA* promoter via proteolytic cleavage. Hence, *mecRI-mecI* system is up regulated enabling full induction of *mecA* gene (Arède et al. 2012). This model has described the essentiality of MecR2 for complete transcription of *mecA* for optimal expression of beta-lactam resistance in MRSA, particularly, PBP2a. Considering the mechanism of *mecA* expression, any substantial inhibition on PBP2a production as observed in our study possibly occurred due to effects of the bioactive fraction on MecR2 or MecR1 or both (Figure 3.15). We suspect that the active molecules from the bioactive fraction are capable of interfering with binding of beta-lactam antibiotic to MecR1 domain therefore preventing the signal transduction for *mecA* activation. Besides, it is also possible that presence of the active molecules from the bioactive fractions have an adverse effect on MecR2 therefore cripples its ability to ensure full induction of *mecA* transcription. In both postulations, we propose that interaction of the bioactive fraction with the receptors as described above hinder full expression of *mecA* and consequently prevent or inhibit production of PBP2a. Figure 3.15 represent a model of *mecA* expression and the possible interference of the bioactive fractions in the mechanism.

The other proposition here is properties of the bioactive fractions are able deactivate PBP2a activity or degrade PBP2a at certain point (Figure 3.15). Older reports have suggested potential of plant products belonging to polyphenols or diterpenes groups in suppressing PBP2a expression (Nicolson et al. 1999; Shimizu et al. 2001; Shiota et al. 2004) but none that actually describes their ability to deactivate PBP2a activity or degrade this particular protein. Although, the mechanism of deactivation or

degradation is elusive, we predict interaction between active components from the bioactive fractions with PBP2a can result in considerable conformational changes in the protein's structure or degrade the protein. Thus, its function to assemble latter stage of cell wall biosynthesis in MRSA is expected to be deprived and following that MRSA becomes susceptible to ampicillin again.

Treatments of ampicillin alone at low concentrations were observed to aggravate PBP2a expression based on high intensity PBP2a bands in Western blot experiment. A low concentration of beta-lactam antibiotic was described to induce processes which foster genetic exchange and antibiotic resistance in *S. aureus* species (Kaplan et al. 2012). This may shed some light on the high expression of PBP2a upon exposure to ampicillin at low concentrations in this study. Furthermore, the expression of PBP2a is restricted to small sub-population in absence of beta-lactam antibiotics and presence of these antibiotics was reported to form a homogenous resistance population (Zapun et al. 2007). Hence, it is likely that the formation of homogenous population in presence of ampicillin contributed to higher expression of PBP2a in our study.

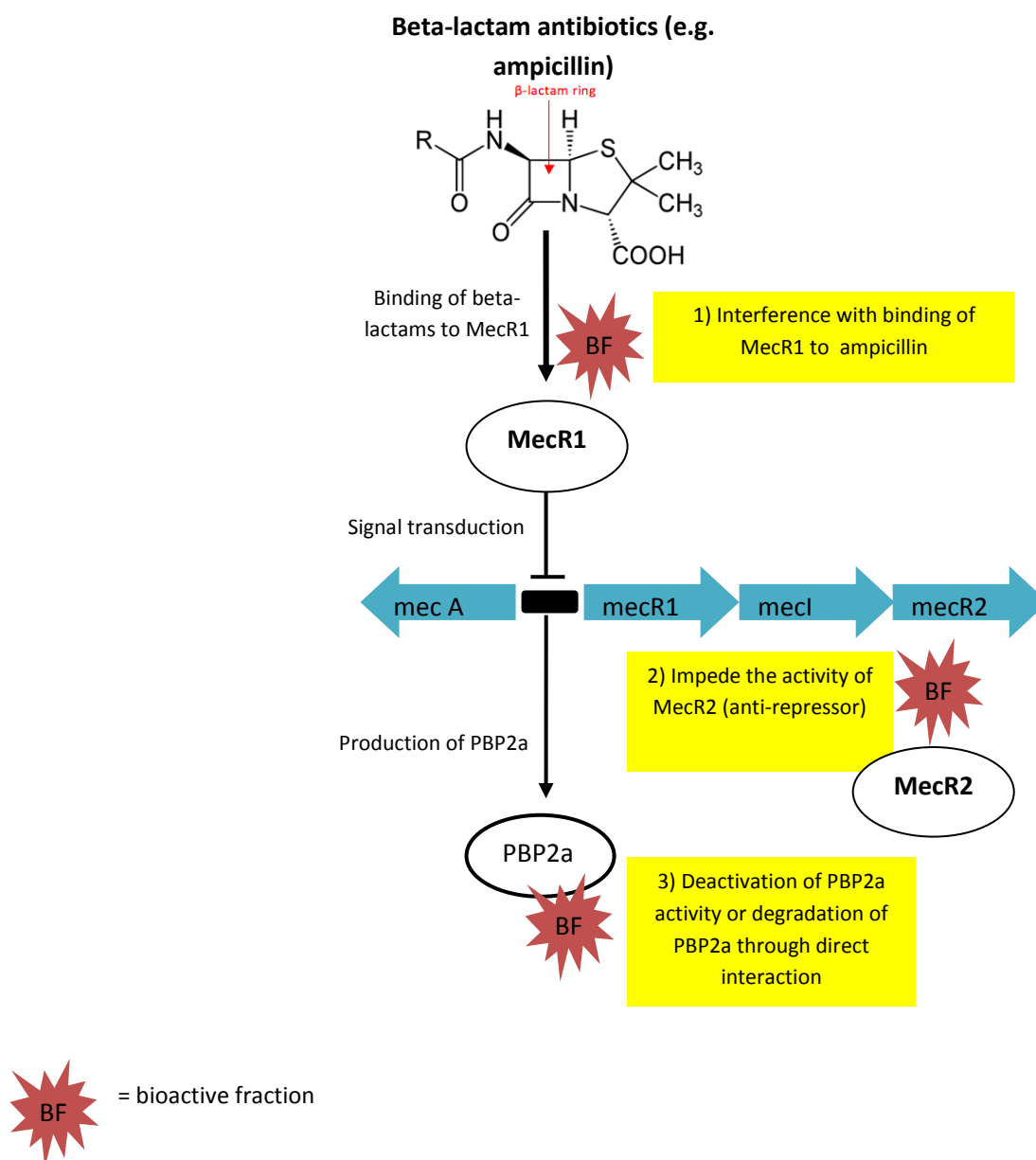


Figure 3.15

A schematic diagram showing induction of *mecA* by *mecR1-mecI-mecR2* regulatory genes in presence of beta-lactam antibiotics. The mode of action of the bioactive fractions (BF) on the PBP2a induction system are postulated due to 1) interference of BF with binding of MecR1 to ampicillin, 2) impediment of MecR2 function by BF or 3) degradation of PBP2a by presence of BF. BF is represented by red stars in the diagram.

3.6 CONCLUSION

The concept of synergism demonstrated by plant test agents from *A. wilkesiana* and *D. grandiflora* in combination with ampicillin was noted potent with > 90% inhibition was observed on MRSA growth. This was based on FIC indices that primarily indicated synergism and rarely indifference. Likewise, growth curves experiment demonstrated that the combination treatments were able to prevent and suppress MRSA growth markedly compared to treatment with one agent alone. Furthermore, the results of growth curve experiments implied that the combination treatments influence bacterial cell division which can be related to DNA replications and other cellular metabolism such as protein production.

MRSA strain used in this experiment carries *mecA* gene which positively encodes for PBP2a which confers resistance to ampicillin. Consistently, MIC ampicillin for this particular strain was 50 µg/ml. This value has exceeded MIC breakpoints for susceptibility to beta-lactam drugs (Brown 2001). Nevertheless, by combining plant test agents from *A. wilkesiana* or *D. grandiflora* with ampicillin, we managed to increase the strain's susceptibility towards ampicillin which was represented by a reduced MIC ampicillin. The restoration of ampicillin's antibacterial potency in the combination treatments indicates that the plant test agents may interact with the resistance factor, PBP2a. Hence, we pursued the study of effects of combination treatments on PBP2a.

In Western blot assay, presence of FC-B and 75EA-L-F10 alone and in combination with ampicillin inhibits production of PBP2a. Therefore, we conclude that one of the mechanisms involved in enhancement of antibacterial effects in combination treatment is inhibition of PBP2a. The mechanism in which PBP2a is affected by the plant test agent is unknown at this stage. Our postulations were that these plant test agents

could interrupt the expression of *mecA* gene or interact with PBP2a and compromised its action. These postulations however, needs further study for confirmation. Besides that, FC-B and 75EA-L-F10 consist of mixture of plant metabolites; hence involvement of several mechanisms in attenuation of PBP2a and the antibacterial effects overall, is undisputable.

CHAPTER 4

INHIBITION OF BIOFILM PRODUCTION IN MRSA

4.1 BACKGROUND

Biofilms are known as surface-adhering bacterial communities encapsulated in extracellular complex comprising DNA, bacterial polysaccharides and proteins by forming a slimy layer (Costerton and Stewart 1999; Mulcahy et al. 2008). They play an intrinsic role in protecting bacterial cells from any fluctuations of the environment including protecting the colonies from any potential antimicrobial agents (Costerton and Stewart 1999). It is well studied that the physiological properties of sessile biofilm populations are different from their planktonic counterparts and these contribute to their better survival within the infected hosts. Biofilm protected bacterial cells present a different mode of growth compared to planktonic cells, and the peculiarity of the mode of growth contributes to manifestation of antibiotic resistances which are distinct from their planktonic counterparts. The biofilm-specific antibiotic resistance mechanisms are 1) delayed or reduced antimicrobial diffusion into biofilm cells, 2) presence of persister cells, 3) antimicrobial destroying enzymes, 4) quorum sensing signaling, and 5) global stress response leading to over expression of antimicrobial resistance (Petrelli et al. 2003; del Pozo and Patel 2007).

The biofilm forming ability of MRSA represents a major factor for nosocomial infections (Costerton et al. 1999). In fact, a recent research has shown that, multi-drug resistant clinical *S. aureus* strains produce more biofilm implying that the biofilm forming ability is crucial for the bacteria survival in presence of antibiotics (Kwon et al. 2008). Similarly, a clinical study in 2012 concluded that *S. aureus* species with biofilm forming properties were observed to express higher antibiotic resistance, thus more difficult to treat (Agarwal and Jain 2012). The currently available antimicrobial agents have not been specifically developed to target bacterial biofilms (del Pozo and Patel 2007). As such, these agents are typically ineffective in treating biofilm-associated

infections (Lynch and Abbanat 2010). Often, removal of the infected devices is the most effective clinical solution in most of the biofilm-related infections cases (Donlan and Costerton 2002; Petrelli et al. 2008). Recently, new approaches such as antimicrobial peptide, bioengineered agents (bacteriophages), ultrasonic treatments, quorum sensing inhibitors, and natural products are being developed for treatment of biofilm-associated infections (Sun et al. 2013).

The steps involved in biofilm production are complex and the process can be categorized into four distinct phases: attachment, accumulation, maturation and dispersal (Christensen et al. 1994). In staphylococcal biofilms, there are two essential steps that have been emphasized; 1) cell-surface attachment, in which the bacteria attach to a surface in order to form colonies and this is also known as the primary attachment step; and 2) cell-cell interaction, which is an accumulative phase where the bacteria form microcolonies for construction of multilayer structure leading to biofilm development (Mack 1999; Götz 2002; Mack et al. 2004; Beenken et al 2004). These two processes are mediated by different types of adhesins. A group of surface-exposed proteins generally termed as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) is responsible for the primary attachment of cells to surface including native tissues and biomaterials (Patti et al. 1994). In accumulative phase, the bacterial cells rely on polysaccharide intercellular adhesin, or poly-N-acetylglucosamine (PIA/PNAG) that facilitates interactions between the cells for formation of microcolonies (Götz 2002). These polysaccharides adhesins are encoded by *icaADBC* genes and they represent a crucial role in staphylococci biofilm formation (O'Neill et al. 2007) (see Figure 4.1a).

MRSA strains however were found to produce *ica* independent biofilm. Deletion of *icaADBC* has no effect on biofilm production in these strains. Study on some MRSA

isolates also showed that PIA/PNAG was not detected in the produced biofilm despite the transcription of *icaADBC*. This is because accumulative phase in development of MRSA biofilm is promoted by protein adhesins namely fibronectin-binding proteins (FnBPs) contrary to PIA/PNAG in MSSA (O'Neill et al. 2007; Szczuka et al. 2013) (Figure 4.1b). FnBPs plays a vital role in intercellular accumulative phase of MRSA biofilm formation and deletion of genes (*fnbA* and *fnbB*) encoding for these proteins resulted in failure to form biofilm (McCourt et al. 2014). Therefore MRSA strains produce proteinaceous biofilm unlike polysaccharide based biofilm formed by MSSA.

The requirement of FnBPs and other surface proteins which function as adhesins in MRSA biofilm production indicates the importance of proteins in promoting the cell-cell interaction during the accumulative phase. The resistant protein, PBP2a is acquired and expressed in MRSA to overcome antimicrobial action of beta-lactam antibiotics and this protein was also found to mediate biofilm formation in MRSA. It was hypothesized that PBP2a facilitates cell-cell interactions in the biofilm production possibly by changing the bacterial cell wall architecture (Berger-Bächi and Rohrer 2002; Pozzi et al. 2012) (Figure 4.1b). Hence, development of anti-biofilm agents that disrupt PBP2a expression not only will be able to reverse methicillin resistance in MRSA but concurrently interfere with biofilm formation. This would be a sensible approach in developing a new treatment for recalcitrant MRSA infections

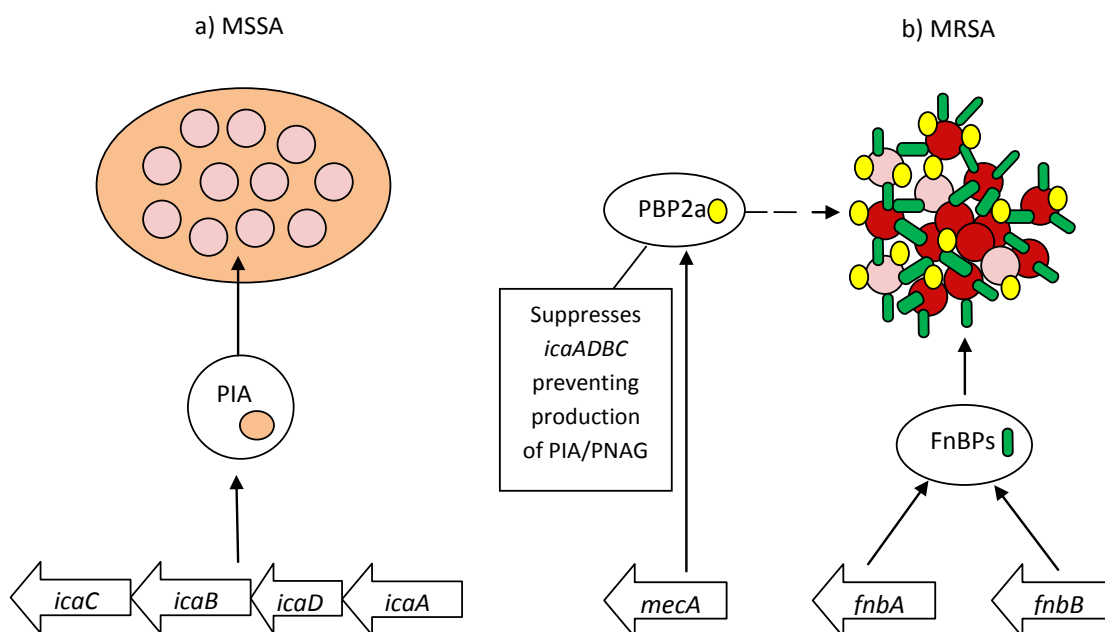


Figure 4.1 Graphic representations of biofilm phenotypes produced by a) MSSA and b) MRSA. MSSA strain forms *ica*-dependent PIA mediated biofilm whereas MRSA strain biofilm is independent of PIA and requires surface proteins such as FnBPs during the accumulative phase. Expression of PBP2a by *mecA* suppresses production of PIA/PNAG and the protein is suspected to exerts direct or indirect (e.g. via altered cell wall architecture) effects on MRSA biofilm production.

In recent reviews, plants products were found to have anti-biofilm activities (Manefield et al. 2002; Abreu et al. 2012; Kim and Park 2013). It is interesting to highlight potentiating of ampicillin's action against MRSA in the presence of bioactive fractions from the plant *A. wilkesiana* and *D. grandiflora* as shown in Chapter 3. Hence, we suspect these bioactive fractions may possess antibacterial properties that are able to inhibit biofilm production. In this chapter, experiments were designed to investigate the effects of the bioactive fractions on MRSA biofilm forming capacity and PBP2a expression in the biofilm matrix.

4.1.1 Biofilm Assays

Biofilm assays in this chapter are divided in two main parts; 1) inhibition of MRSA biofilm production and 2) microtiter attachment assay. Inhibition of biofilm production assay provides information on final mass of biofilm production upon treatments. Whilst, microtiter attachment assay will indicate if the antimicrobial agent is preventing cell-surface association which is the first step involved in biofilm development (O'Toole et al. 2000; Watnick and Kolter 2000). In both assays, quantification of biofilms and cell attachment to surface were done by measuring optical density (OD) of crystal violet staining using an enzyme immunosorbent assay reader (Stepanović et al. 2000; Stepanović et al. 2007). The crystal violet staining works according to the ability of the bacterial cell wall to reserve the dye during the staining procedure. Besides that, crystal violet dye also stains extracellular matrix which is useful in study of biofilms (Welch et al. 2012). Christensen et al. (1982 & 1985) first described the staining of bacterial film lining culture with a cationic dye and measuring of the OD using a spectrophotometer. The results although qualitative in nature, lack of completeness because only biofilm on the well's bottom was measured (Stepanović et al. 2000). To improve the objectivity and accuracy of the assay, a modified test with fixing of the bacterial cells, staining with crystal violet, decolourizations of the stain, and finally measuring the OD as described previously (Stepanović et al. 2000; Overhage et al. 2008; Durham-Colleran et al. 2010; Mataraci and Dosler 2012) was employed in this study. Decolourization of the stain leeches the dye and the solvent gathers the solubilised dye in the bottom of the well. As such, the cell film lining attached around the well are not discriminated.

Commonly used media to grow biofilm of *Staphylococcus* species is tryptic soy broth (TSB) supplemented with glucose (Stepanović et al. 2007; Overhage et al. 2008; Mataraci and Dosler 2012). According to Fitzpatrick et al. (2006), MRSA strains are

more likely to form biofilm in media supplemented with glucose. A following study in 2009, explained that biofilm are normally found to be colonizing medical device like catheters and heart valve that are in direct contact with blood. Based on a previous report study, *in vitro* biofilm formation in *S. aureus* lineage is positively correlated to physiological concentration of glucose (Croes et al. 2009).

4.1.2 Study of biofilm phenotype and PBP2a latex agglutination

Biofilm study in this chapter is also extended to determine the biofilm phenotype produced by MRSA ATCC 43300. Two chemical agents, proteinase K and sodium metaperiodate were used in previous studies to determine the biofilm phenotype. Based on these studies, dispersal of biofilm caused by either of this agent indicates whether the biofilm is protein or polysaccharide based (Holland et al. 2011; Pozzi et al. 2012). Proteinase K is an enzyme that has broad specificity and is able to degrade many proteins at native state. It predominantly cleaves peptide bonds in protein leading to its breakdown subsequently disperse proteinaceous biofilm. Sodium metaperiodate degrades PIA-dependent biofilm due to its ability to oxidize and cleaves cis-diols in carbohydrate sugars.

PBP2a latex agglutination assay is usually used for detection of PBP2a from MRSA cultures grown on plate. In a PBP2a latex agglutination kit, the latex reagent is sensitized with monoclonal antibody against PBP2a. Therefore, when the extracted supernatant is mixed with latex reagent, a visible clumping is formed indicating presence of PBP2a. An elaborate study by Pozzi et al. (2012) demonstrated that biofilm produce by an induced –methicillin resistant strain carrying *mecA* gene was reduced when the strain was grown in presence of monoclonal PBP2a antibody suggesting the role of PBP2a in MRSA biofilm development. Hence, a PBP2a latex agglutination test was

conducted to study effects of the bioactive fractions on PBP2a level in MRSA biofilms. A commercially available kit is used for the test and this kit was reported suitable to detect PBP2a rapidly in less complicated manner (Doern et al. 1994; Cavassini et al. 1999). Earlier, a PBP2a latex agglutination kit was used to evaluate inhibition of PBP2a by plant metabolites and the method was described to yield semi-quantitative results based on intensity of the agglutination (Zhao et al. 2001).

4.2 AIMS AND OBJECTIVES

Experiments in this chapter were aimed to investigate anti-biofilm properties of the bioactive fractions FC-B and 75EA-L-F10 alone and combination with ampicillin. The specific objectives is to investigate the effects of the selected combination treatments on biofilm production in MRSA

4.3 MATERIAL AND METHODS

Based on results from chapter 3, two bioactive fractions FC-B and 75EA-L-F10 (from *A. wilkesiana* and *D. grandiflora* respectively) were selected for this biofilm study. These two plant test agents demonstrated synergism with ampicillin by reducing MIC of ampicillin the most compared to the other plant test agents used in this study. In this chapter, these bioactive fractions and ampicillin were tested at MIC and sub-MICs to investigate the effect of these agents on MRSA biofilms. The MIC values of the bioactive fractions and ampicillin used in this biofilm study were the MIC against MRSA growth in planktonic state obtained from experiments in Chapter 3 (broth microdilution assay).

4.3.1 Microtiter attachment assay

This assay was performed based on previously reported method with a minor modification (Overhage et al. 2008). A 96-well microtiter plate prepared with ampicillin alone, bioactive fraction alone and in combination of both as explained in section 4.3.4 kinetic growth curve assay (Chapter 3). MRSA culture that was grown 24 hours prior to experiment was diluted with TSB (Hi-Media, India) + 1 % glucose (Merck, Germany) and added to these wells containing different treatments. The inoculums size in each well was 1×10^7 CFU/ml in final volume 200 μ l. The plate was incubated for 1 hour at 35°C. After the incubation, quantification of cell attachment to surface was done based on methods described in section 4.3.3. Experiment was done in triplicates on three separate occasions.

4.3.2 Inhibition of biofilm production

This experiment was conducted according to Mataraci and Dosler 2012 with a slight modification. Similar to the microtiter attachment assay, a 96-well microtiter plate was prepared with bioactive fraction alone, ampicillin alone and in combinations. MRSA suspension that was grown 24 hours prior to experiment was diluted with TSB + 1 % glucose to correspond to 1×10^5 CFU/ml upon inoculation in final volume of 200 μ l of each well. The plate was incubated for 24 hours at 35°C. After the incubation, quantification of biofilm production was done based on methods described in section 4.3.3. Experiment was done in triplicates on three separate occasions.

4.3.3 Quantification of cell attachment and biofilm production

Quantification of cells attachment in microtiter attachment assay and inhibition of biofilm production assay were determined via crystal violet staining method (Durham-Colleran et al. 2010). After the incubation, media was aspirated and the wells of the microtiter plate were washed three times with 250 μ l of physiological buffered saline (PBS) to remove unattached bacteria and left to dry. Upon drying, 200 μ l of 99% methanol (Fisher Scientific Chemicals, USA) was added into each well for fixation. Then, the solvent was removed and plates were allowed to dry for 15 minutes. Following that, the wells were stained with 0.1% crystal violet (v/v in water) (R&M Chemicals, UK) for 5 minutes. Using tap water, the excess stain was discarded gently and plates were air dried. Stain was dissolved by adding 200 μ l of 95% ethanol (Fisher Scientific Chemicals, USA) with the plates left on orbital shaker (Fisher Scientific 260300 Ocelot Orbital, USA) at 50 rpm for 30 minutes. Finally the OD was read at 595 nm using a multimode plate reader (Varioskan Flash, Thermo Scientific, USA). Percentage of MRSA cell-surface attachment and biofilm production was determined by measuring absorbance of every

treated well at 595 nm subtracting absorbance of control MRSA (treatment free) which was used as 100 % growth reference.

4.3.4 Determination of MRSA biofilm phenotype

The experiment was conducted as described previously (Mack et al. 1992; Holland et al. 2011). An overnight MRSA culture was diluted with TSB + 1 % glucose and 30 µl aliquot of the suspension was added to the wells of microtiter plate. The inoculums size in each well was 1×10^7 CFU/ml when the final volume was made up to 200 µl by adding the growth media. Plate was incubated for 24 hours at 35°C. After incubation, media was removed and the wells were washed with 100 µl PBS. The microtiter plate was then allowed to dry for 15 minutes. Proteinase K (50 µl, 100 µg/ml) (Sigma, USA) or sodium metaperiodate (50 µl, 10 mM) (Sigma, USA) was added to the wells and the microtiter plate was further incubated for 2 hours at 35°C. Following incubation, the chemical agents were removed by washing the wells with sterile distilled water (100 µl) and the plate was dried at 65°C for 1 hour. The absorbance of adhered biofilm was measured using the methods described in section 4.3.3. Experiment was done in triplicates on three separate occasions.

4.3.5 PBP2a latex agglutination test on MRSA biofilm

Prior to the latex agglutination test, MRSA was cultured in 50 mm diameter petri dishes in 10 ml of TSB + 1 % glucose supplemented with MIC and sub-MICs of ampicillin alone, bioactive fraction alone and in combination of both. The petri dishes were incubated for 24 hours at 35°C. After incubation, the broth was carefully removed and 500 µl PBS was added to the petri dishes. Using a sterile 5 µl inoculating loop, the biofilm layer was scraped off just to fill the internal diameter (gives approximately 1.5×10^9 CFU/ml). The

obtained bacterial biofilm was processed according to MRSA screening kit (Cat. no. DR900A Denka Seiken, Japan) manufacturer's instructions in order to detect presence of PBP2a.

Briefly, PBP2a in the obtained bacterial biofilm was extracted by using extraction reagents (0.1 mol/L NaOH and 0.5 mol/L KH₂PO₄) found in the assay kit. The extracts then were mixed with test latex that was sensitized with a monoclonal antibody against PBP2a. Presence of PBP2a was indicated by formation of agglutination in the extract. Semi-quantitative estimation of PBP2a agglutination strength in the extract was done based on protocols described in Zhao et al. (2001) in which the agglutination strength was observed and scored between + and + + +, where the control latex which showed no reactivity in the absence of PBP2a is considered as "-". The control latex is sensitized with a monoclonal antibody of the same IgG subclass but against a human protein showed no reactivity with proteins of *S. aureus*.

4.3.6 Statistical analysis

Results for biofilm attachment and inhibition assays were shown as means \pm standard deviation of three independent experiments. A one-way analysis of variance with Bonferroni multiple comparison tests was used to compare difference between the control and combination treatment group biofilms. A *P* value of < 0.001 was taken as statistically significant (Mataraci and Dosler 2012).

4.4 RESULTS

4.4.1 FC-B and 75EA-L-F10 prevent cell-surface attachment in MRSA

MRSA cell-surface interaction was studied by growing the cultures in the presence of ampicillin ranging from $1/64 \times \text{MIC}$ ($0.78 \mu\text{g/ml}$) to MIC ($50 \mu\text{g/ml}$). Overall, ampicillin showed a modest reduction in MRSA's attachment to the well's surface (see Figure 4.2). At all tested concentrations, $>50\%$ of cells were found to attach and remain on the microtiter plate. The percentage (%) of attachment appeared to be concentration dependent as the highest cell-surface attachment (81.16%) was recorded for $1/64 \times \text{MIC}$ of ampicillin and lowest for MIC of ampicillin (62.20).

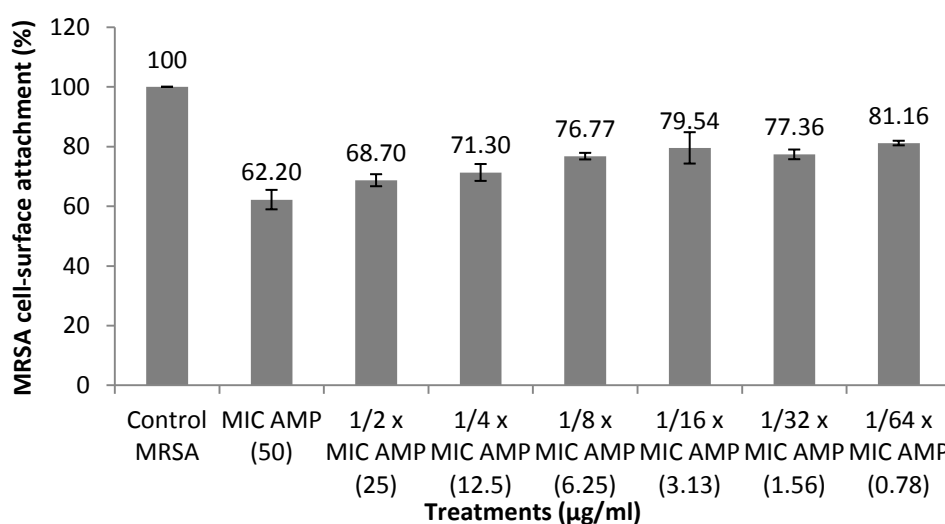


Figure 4.2 Effects of ampicillin on MRSA cell-surface attachment. Three wells were used for each treatment. Experiment is representative of three independent tests, and error bars indicate the standard deviation. All difference between control and treated MRSA were statistically significant ($p < 0.001$) (MIC= minimum inhibitory concentration, AMP= ampicillin).

Bioactive fractions, FC-B and 75EA-L-F10 were evaluated at concentrations ranging from MIC to 1/16 x MIC. Briefly, both fractions at their MIC were found to markedly reduce cells attachment to the growth surface.

Figure 4.3 shows % of MRSA cell-surface attachment in presence of FC-B. At the MIC FC-B (3.00 mg/ml), less than 10% of cells attached to the surface. FC-B inhibits attachment of MRSA cells to the well surface in a concentration-dependant manner. The inhibitory action on cell- surface attachment was observed to be considerably potent at 1/2 x MIC FC-B (1.50 mg/ml, 28.56%) and 1/4 x MIC (0.75 mg/ml, 42.59%). Nevertheless, at lower concentrations, the effect of FC-B was similar to ampicillin's (Figure 4.3).

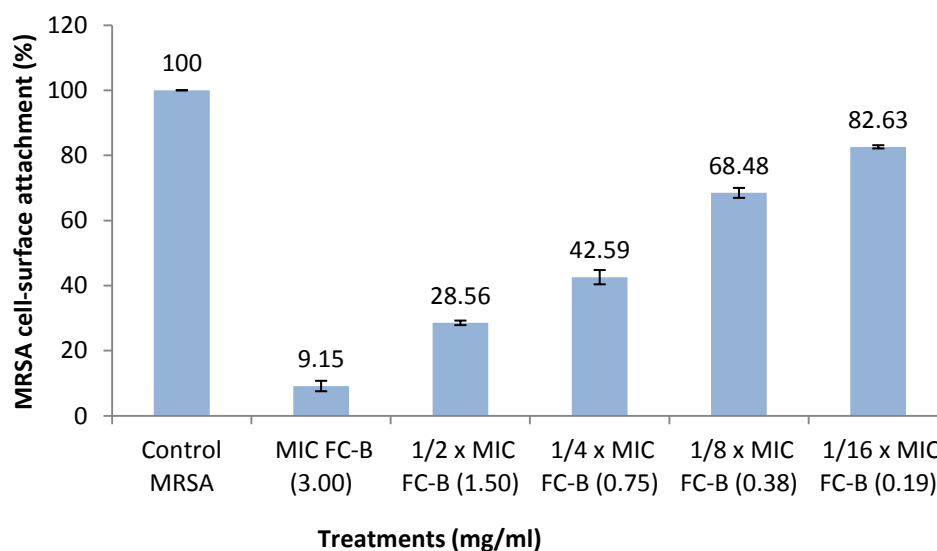


Figure 4.3 Effects of FC-B on MRSA cell-surface attachment. Three wells were used for each treatment. Experiment is representative of three independent tests, and error bars indicate the standard deviation. All difference between control and treated MRSA were statistically significant ($p < 0.001$) (MIC= minimum inhibitory concentration).

Based on Figure 4.4, results for MRSA cell-surface attachment revealed that the presence of 75EA-L-F10 at MIC (0.75 mg/ml) markedly inhibits MRSA cells from attaching to the growth surface as shown by a mere 5.33% of cell attachment. At 1/2 x MIC 75EA-L-F10 (0.38 mg/ml), this fraction was still effective in preventing cell attachment. 75EA-L-F10 shows similar concentration-dependant inhibitory effects as FC-B. At lower concentrations, 75EA-L-F10 produces similar magnitude of inhibition as ampicillin.

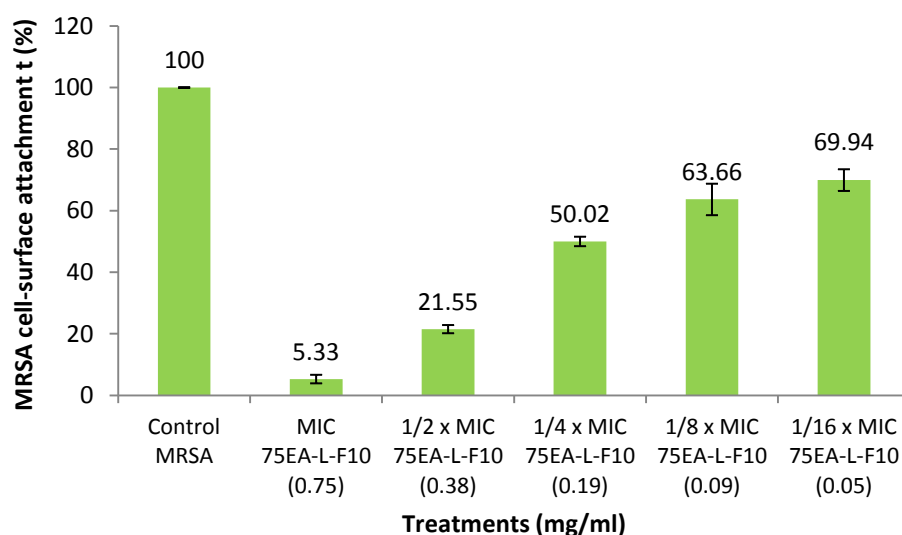


Figure 4.4 Effects of 75EA-L-F10 on MRSA cell-surface attachment. Three wells were used for each treatment. Experiment is representative of three independent tests, and error bars indicate the standard deviation. All difference between control and treated MRSA were statistically significant ($p < 0.001$) (MIC= minimum inhibitory concentration).

The combined effects of the bioactive fractions, FC-B and 75EA-L-F10 and ampicillin in preventing cell-surface attachment were studied by investigating two combination treatments; $1/32 \times \text{MIC}$ ampicillin + $1/4 \times \text{MIC}$ FC-B and $1/64 \times \text{MIC}$ ampicillin + $1/4 \times 7\text{EA-L-F10}$. Ampicillin only inhibits less than 23% cells from attaching to the surface but presence of FC-B and 75EA-L-F10 inhibit more than 47% and 50% cells attachments (Figure 4.5). In combination treatment ampicillin + FC-B, a significantly increased inhibitory action in preventing cell-surface attachment was observed (75.8%). Treatment of 75EA-L-F10 and ampicillin produced similar results (48.8%) as fraction 75EA-L-F10 alone (50%) (Figure 4.5).

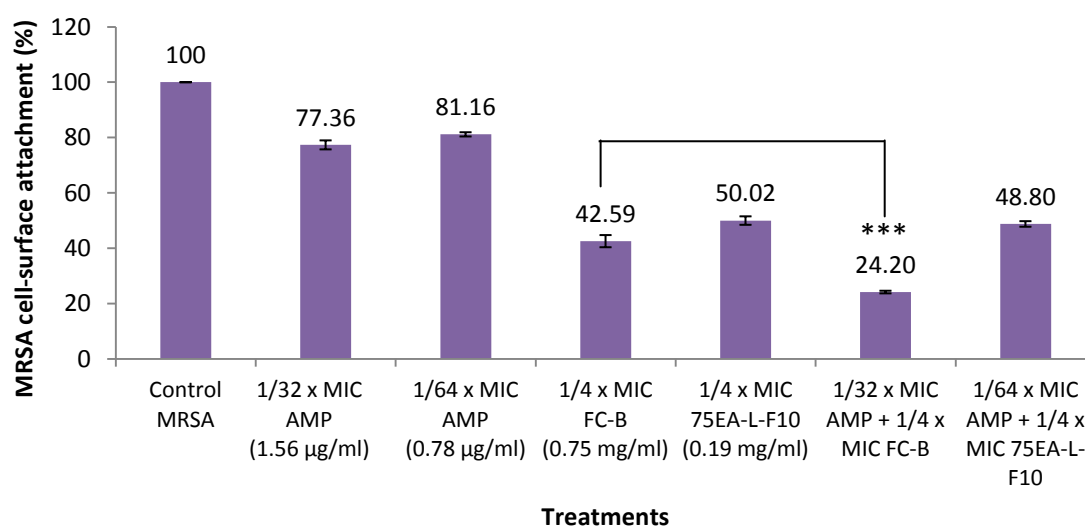


Figure 4.5 Effects of ampicillin, FC-B and 75EA-L-F10, alone and in combinations. Three wells were used for each treatment. Experiment is representative of three independent tests, and error bars indicate the standard deviation. All difference between control and treated MRSA were statistically significant ($p < 0.001$) (AMP= ampicillin, MIC= minimum inhibitory concentration, *** = significance difference between the plant test agent alone and in combination).

4.4.2 Inhibition of MRSA biofilm production by FC-B and 75EA-L-F10

Effects of ampicillin, FC-B and 75EA-L-F10 on MRSA biofilm production were investigated. The results demonstrated that MIC of ampicillin (50 µg/ml) markedly suppressed MRSA biofilm production with just 15.51% of biofilm formed compared to untreated MRSA culture (Figure 4.6). Biofilm production in different concentrations of ampicillin was shown to be concentration-dependant and at 1/2 x MIC ampicillin (25 µg/ml), the antibiotic inhibited about 35% of biofilm production. As ampicillin concentration was lowered, the biofilm production increased and eventually at 1/64 x MIC ampicillin (0.78 µg/ml), the formation of biofilm was hardly affected (92.3%). This results indicated that a high concentration of ampicillin (MIC = 50 µg/ml) is needed to suppress MRSA biofilm formation, nevertheless, this concentration did not totally inhibit the biofilm synthesis.

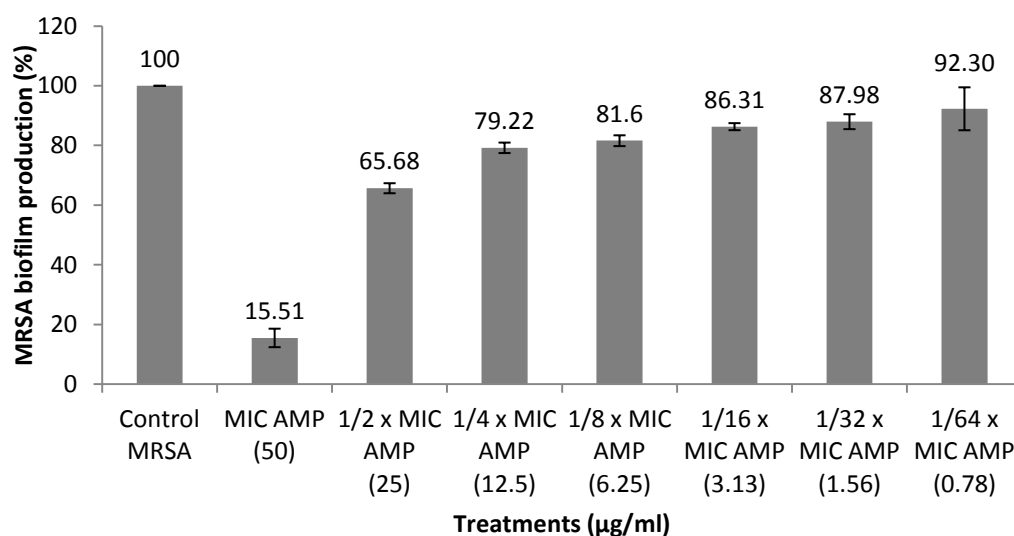


Figure 4.6 Effects of ampicillin on MRSA biofilm production. Three wells were used for each treatment. Three wells were used for each treatment. Experiment is representative of three independent tests, and error bars indicate the standard deviation. All difference between control and treated MRSA were statistically significant ($p < 0.001$) (AMP= ampicillin, MIC= minimum inhibitory concentration).

The inhibitory activity of FC-B on MRSA biofilm formation is concentration dependant. Hence, the highest tested concentration (MIC= 3 mg/ml) was observed to result in the strongest inhibition in which the biofilm production was only 18.44% (Figure 4.7). At sub-MICs from 1/2 x MIC (1.50 mg/ml) to 1/8 x MIC (0.38 mg/ml), a considerable inhibition on MRSA biofilm (> 2-fold) was achieved with the % of inhibition ranged from 46.15% to 69.78%. Further reduction in concentration (1/16 x MIC= 0.19 mg/ml) appeared to have weakened anti-biofilm action, as the biofilm production reached 69.24% (~70%). Comparable to results of ampicillin treatment, FC-B exhibited strong anti-biofilm activity at MIC level and likewise the high concentration was unable to suppress the biofilm production entirely.

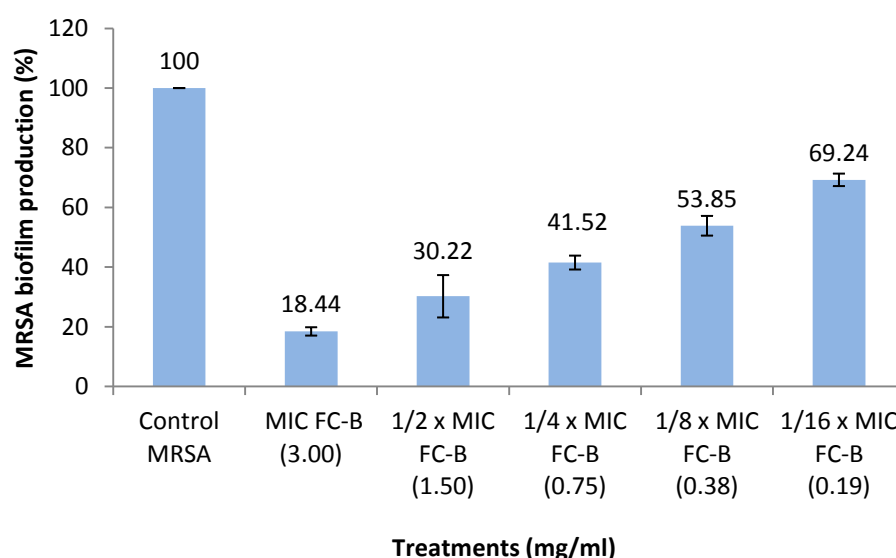


Figure 4.7 Effects of FC-B on MRSA biofilm production. Three wells were used for each treatment. Three wells were used for each treatment. Experiment is representative of three independent tests, and error bars indicate the standard deviation. All difference between control and treated MRSA were statistically significant ($p < 0.001$) (MIC= minimum inhibitory concentration).

Figure 4.8 shows production of MRSA biofilm in presence of 7EA-L-F10. MRSA biofilm production falls in between 33.90 to 52.19 % in treatment of 75EA-L-F10 (for all

tested concentrations) which shows small variability. At MIC 75EA-L-F10 (0.75 mg/ml) a moderate activity against MRSA biofilm formation (33.90%) was observed which was less effective compared to ampicillin at the respective MIC. From this data, it is reasonable to conclude that presence of 75EA-L-F10 generally has moderate action on MRSA biofilm production.

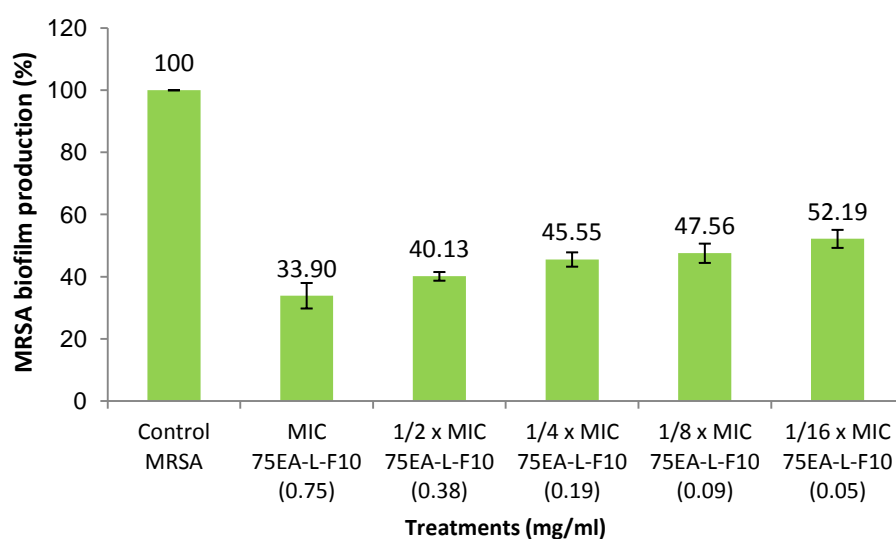


Figure 4.8 Effects of 75EA-L-F10 on MRSA biofilm productions. Three wells were used for each treatment. Experiment is representative of three independent tests, and error bars indicate the standard deviation. All difference between control and treated MRSA were statistically significant ($p < 0.001$) (MIC= minimum inhibitory concentration).

The effects of combination treatment between the bioactive fractions and ampicillin on biofilm production were studied by investigating two combination treatments; $1/32 \times \text{MIC}$ ampicillin + $1/4 \times \text{MIC}$ FC-B and $1/64 \times \text{MIC}$ ampicillin + $1/4 \times 7\text{EA-L-F10}$. Treatment with ampicillin alone as observed earlier has very weak activity on the biofilm formation. Presence of the bioactive fractions at $1/4 \times \text{MIC}$ exhibited appreciable activity against biofilm production, where the biofilm production was at least 2-fold lower compared to control MRSA (Figure 4.9). In combination treatments, the production of biofilm was generally lower compared to treatment with the bioactive fractions alone. However, there was no significance reduction in biofilm formation between treatment of FC-B alone (41.52%) and in combination with ampicillin (32.7%). Whilst combination treatment of 75EA-L-F10 and ampicillin (29%) showed a significant difference in biofilm production compared to treatment with 75EA-L-F10 alone (45.55%) (Figure 4.9).

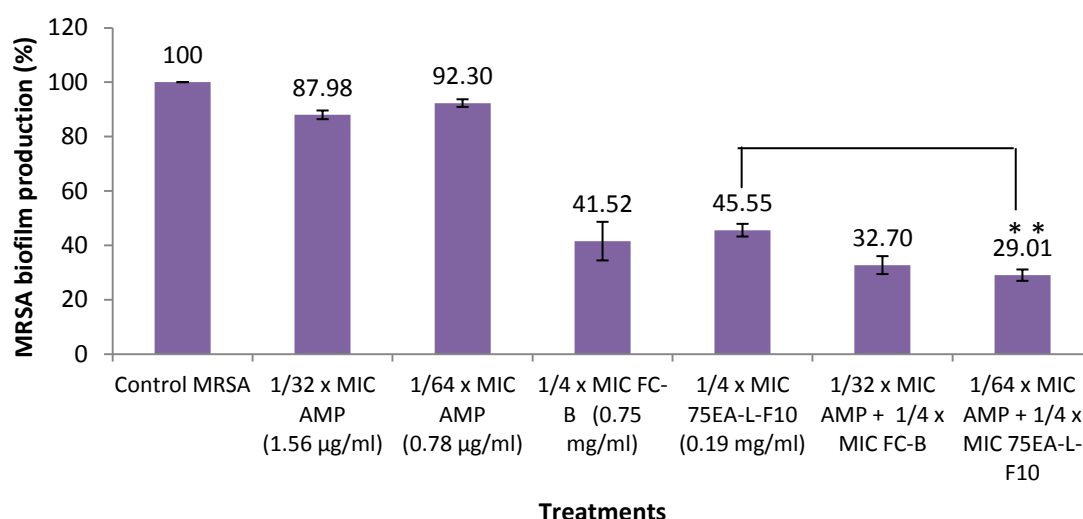


Figure 4.9 Effects of ampicillin alone, FC-B alone, 75EA-L-F10 alone and in combinations on MRSA biofilm production. Three wells were used for each treatment. Experiment is representative of three independent tests, and error bars indicate the standard deviation. All difference between control and treated MRSA were statistically significant ($p < 0.001$) (AMP= ampicillin, MIC= minimum inhibitory concentration, ** = significance difference between the plant test agent alone and in combination).

4.4.3. High dispersal of MRSA biofilm by proteinase K

The cultures treated with proteinase K and sodium metaperiodate were stained with crystal violet to observe the extent of biofilm disintegration. This was carried out by measuring the OD of biofilm that was still intact in the wells after the treatment. The control MRSA (untreated) wells were stained heavily in purple by crystal violet showing formation of biofilm (Figure 4.10a). Both proteinase K and sodium metaperiodate affected the integrity of MRSA biofilm structure. Analysis of the data revealed more than 77% of biofilm was dispersed in presence of proteinase K but in sodium metaperiodate treated cultures only 22.41% of the biofilm was degraded. Thus, implies that biofilm produced by MRSA strain used in this study expressed a higher level of protein compared to polysaccharide.

The image of crystal violet staining of biofilms treated with proteinase K shows that the staining was only found around the periphery of the well's bottom (like a ring) and the middle section is clear. This indicates the film lining has been largely dispersed. In contrast, wells that contained biofilm treated with sodium metaperiodate were entirely stained by crystal violet suggesting the biofilm structure is still intact. When compared to control MRSA, wells containing sodium metaperiodate treated biofilm were less intense (Figure 4.10a). Thus, implied that sodium metaperiodate may affect integrity of the MRSA biofilms but not as extensive as proteinase K. Consistent with visual observation, the OD of control MRSA was the highest (1.06), followed by sodium metaperiodate (0.78) and proteinase K (0.24) (Figure 4.10b).

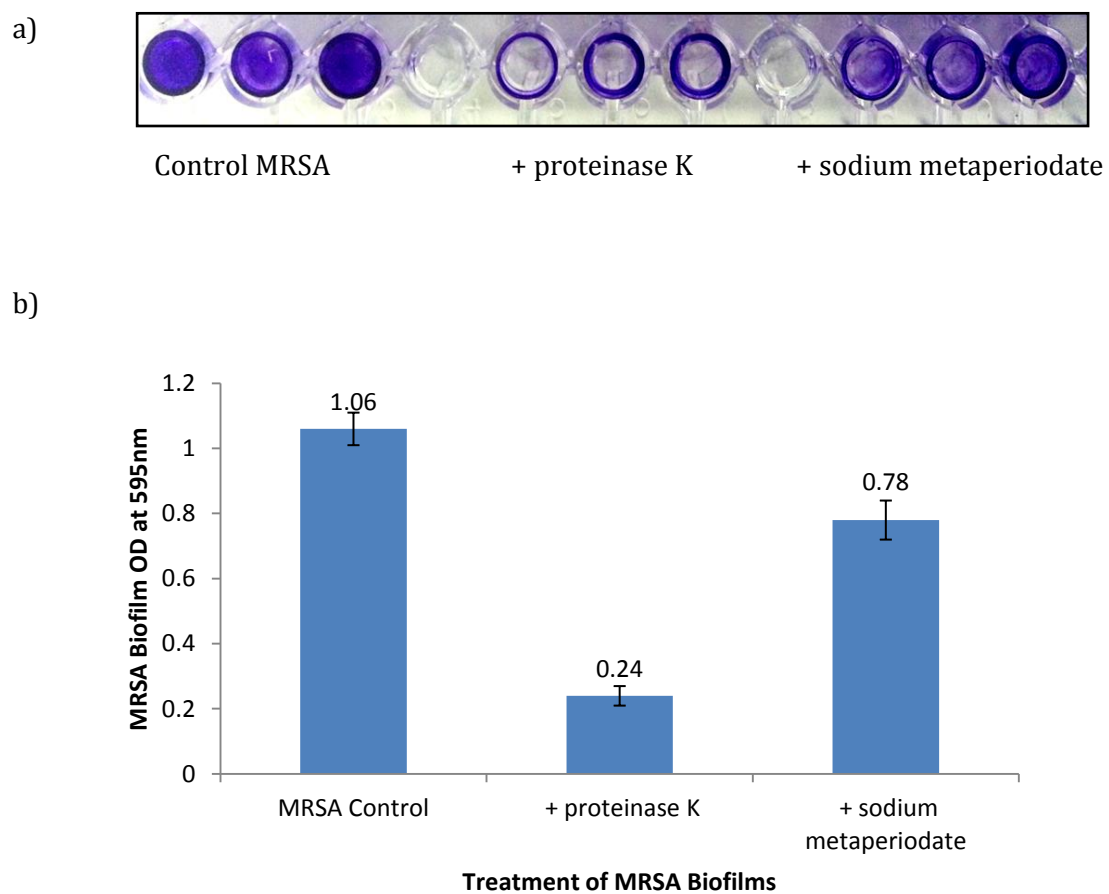


Figure 4.10 a) Crystal violet staining of MRSA ATCC 43300 biofilms in the presence of proteinase K and sodium metaperiodate in microtiter plate. b) Bar graph representing OD of biofilms after treatment with proteinase K and sodium metaperiodate. Three wells were used for each treatment. Experiment is representative of three independent tests, and error bars indicate the standard deviation.

4.4.4 FC-B and 75EA-L-F10 reduced PBP2a in MRSA biofilm

Presence of PBP2a was detected by formation of agglutination when the supernatant from the biofilm was mixed with sensitized test latex. Some tested aliquots appeared turbid during the assay, but turbidity alone without agglutination is not an indication of PBP2a presence. The degree of agglutination was determined by visual observation compared to a negative control (no agglutination). Figure 4.11 shows moderate agglutination indicating presence of PBP2a in control MRSA. The agglutination was predominantly seen around the periphery of the aliquot. Biofilms isolated from treatment of MIC of ampicillin (50 µg/ml) formed a stronger agglutination compared to control MRSA. Lower concentrations of ampicillin ($1/32 \times \text{MIC} = 1.56 \mu\text{g/ml}$ and $1/64 \times \text{MIC} = 0.78 \mu\text{g/ml}$) were found to have similar agglutination strength as the control (see Figure 4.11).

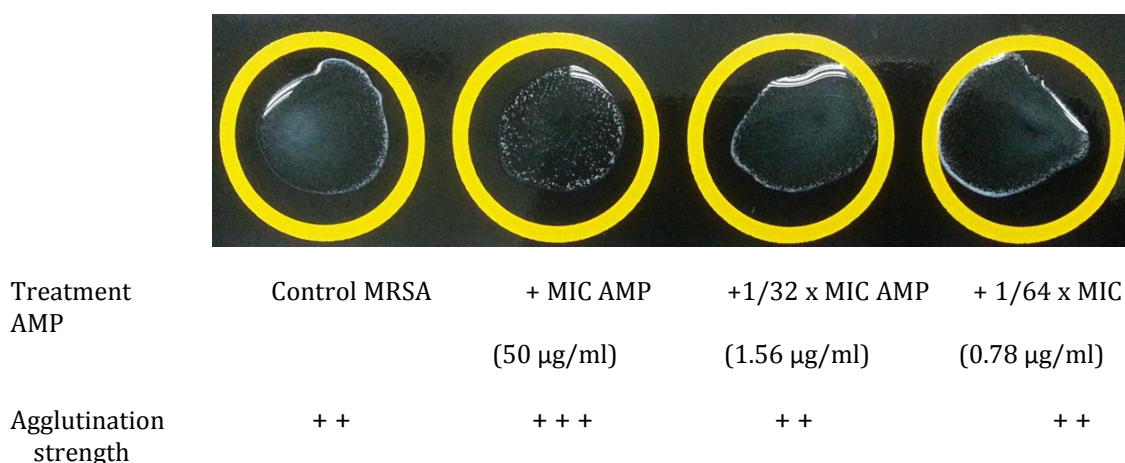
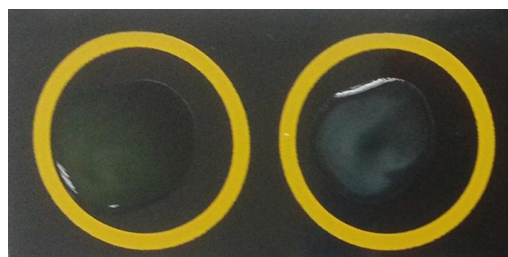


Figure 4.11 Formation of latex agglutination in supernatant of MRSA biofilms obtained from cultures grown in presence of ampicillin. The presence of white agglutination indicates positive for presence of PBP2a. Intensity of agglutination was observed and scored between + and + + +, where the control latex which showed no reactivity in the absence of PBP2a is considered as “-” (interpretation: + + + strong agglutination, + + moderate agglutination against turbid background, + slight agglutination against turbid background, - no agglutination, AMP= ampicillin, MIC = ampicillin, n=3).

Table 4.1 presents the results for all the treatments studied including the bioactive fractions (FC-B and 75EA-L-F10). Treatment with FC-B and 75EA-L-F10 reduced the agglutination for PBP2a compared to untreated MRSA. This indicates the presence of PBP2a in the biofilm but at a lower amount compared to control. The only treatment that abolished the agglutination is the combination of ampicillin (1/32 x MIC) and FC-B (1/4 x MIC) (see Figure 4.12). This is an interesting observation as the combination also presented lowest cell-surface attachment (Figure 4.5).

Table 4.1 Semi-quantitative estimation of PBP2a occurrence in MRSA biofilms isolated from cultures exposed to different treatments. Intensity of agglutination was observed and scored between + and + + +, where the control latex which showed no reactivity in the absence of PBP2a is considered as “-” (interpretation: + + + strong agglutination, + + moderate agglutination against turbid background, + slight agglutination against turbid background, - no agglutination, MIC = ampicillin, n=3).

Treatments	Intensity of PBP2a agglutination
Control MRSA	+ +
MIC ampicillin (50 µg/ml)	+ + +
1/32 x MIC ampicillin (1.56 µg/ml)	+ +
1/64 x MIC ampicillin (0.78 µg/ml)	+ +
1/4 x MIC F-CB (0.75 mg/ml)	+
1/8 x MIC F-CB (0.38 mg/ml)	+
1/16 x MIC F-CB (0.19 mg/ml)	+
1/4 x MIC 75EA-L-F10 (0.19 mg/ml)	+
1/8 x MIC 75EA-L-F10 (0.09 mg/ml)	+
1/16 x MIC 75EA-L-F10 (0.5 mg/ml)	+
1/32 x MIC ampicillin + 1/4 x MIC F-CB	-
1/64 x MIC ampicillin + 1/4 x MIC 75EA-L-F10	+



1/32 x MIC AMP + 1/64 x MIC AMP +
1/4 x MIC FC-B 1/4 x MIC 75EA-L-F10

Figure 4.12 Formation of PBP2a latex agglutination in supernatant of MRSA biofilms obtained from cultures grown in combination treatment. a) 1/32 x MIC ampicillin + 1/4 x MIC FC-B- showing no agglutination indicating absence of PBP2a and b) 1/64 x MIC ampicillin + 1/4 x MIC 75EA-L-F10- showing very mild agglutination against turbid background indicating occurrence of low level of PBP2a. (AMP= ampicillin, MIC= minimum inhibitory concentrations, n=3).

4.4.5 Summary of results

In this chapter, the effects of FC-B and 75EA-L-F10 alone and in combination with ampicillin on MRSA biofilm forming capacity were studied. The results of the measured parameters that were affected by the treatments are summarized in Table 4.2.

The biofilm study, suggested that the bioactive fractions alone are able to reduce MRSA biofilm production more than 2-fold compared to control MRSA. The combination treatments of FC-B and ampicillin however, were observed not to result in significant inhibition MRSA biofilm production compared to treatment with the fraction alone. Whereas, a significance improvement was recorded in combination treatment of 75EA-L-F10 and ampicillin compared to treatment with this fraction alone in suppressing biofilm production. Results from microtiter attachment assay revealed that FC-B and 75EA-L-F10 were able to obstruct MRSA cell-surface attachment with inhibition ranging in between 50-60%. The combination treatments similarly, demonstrated prevention of cell-surface attachment with significant reduction was observed between 1/32 x MIC ampicillin + 1/4 x MIC FC-B and FC-B alone. The PBP2a latex agglutination suggested

that presence of FC-B and 75EA-L-F10 reduced the level of PBP2a in the biofilm matrix. Interestingly, PBP2a was not detected in biofilm treated with the combination treatment 1/32 x MIC ampicillin + 1/4 x MIC FC-B which has lowest cell attachment %.

Table 4.2 Summary of results of experiments conducted to study mechanism of anti-MRSA action exhibited by bioactive fraction FC-B and 75EA-L-F10 alone and in combination with ampicillin (MIC= minimum inhibitory concentration, - = not expressed or absent, * = significant difference $P < 0.001$).

Treatments	Experiments		
	Cell- surface attachment (%)	Biofilm production (%)	PBP2a agglutination in MRSA biofilm (agglutination strength)
Control MRSA	^a 100	^a 100	++
1/32 x MIC ampicillin	77.36 ±1.6	87.98 ±1.6	++
1/4 x MIC FC-B	42.59 ±2.2	41.52 ±7.1	+
1/32 x MIC ampicillin + 1/4 x MIC FC-B	24.20 ±0.5***	32.70 ±3.3	-
1/64 x MIC ampicillin	81.16±0.7	92.30 ±1.4	++
1/4 x MIC 75EA-L-F10	50.02 ±1.5	45.55 ±2.3	+
1/64 x MIC ampicillin + 1/4 x MIC 75EA-L-F10	48.80 ±1.1	29.01±2.0**	+

^aTreatment free MRSA control was used as 100% growth reference to determine % of MRSA cell-attachment and biofilm production.

The biofilm studies also implied that there is positive correlation between % cell-surface attachment and final biofilm production for treatments listed in Table 4.2 (see figure 4.13).

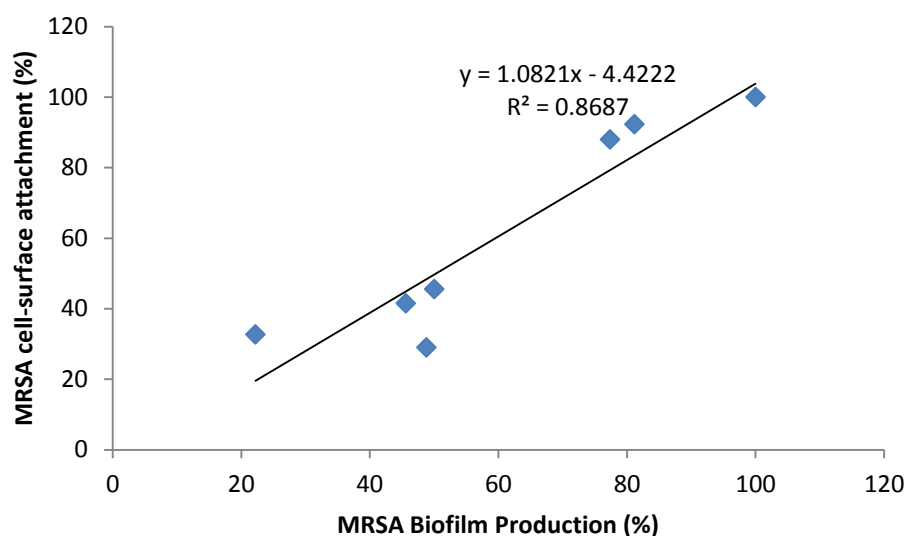


Figure 4.13 A linear regression curve showing relationship between MRSA cell surface attachment and biofilm production for treatments of ampicillin, FC-B and 75EA-L-F10 alone and in combination as described in Table 4.2.

4.5 DISCUSSION

The emergence of MRSA as a globally important human pathogen is attributed to combination of multiple factors such as antibiotic resistance, enzyme and toxin production, biofilm formation, and immune evasion capacity (Pozzi et al. 2012). Biofilm especially possesses threat in hospital settings where the usage of medical implanted devices is common for treatments and diagnostics. Reviews from past have suggested that plant products have antibacterial properties including anti-biofilm activities. Besides, synergistic effects achieved by applying combination of plant products with conventional antibiotics can be employed to suppress the virulent factors in MRSA and consequently overcome the resistance problems (Sibandah and Okoh 2007; Schelz et al. 2010; Abreu et al. 2012). Our research in this chapter focused on the effects of bioactive fractions isolated from *A. wilkesiana* and *D. grandiflora* on biofilm production in MRSA.

4.5.1 Bioactive fractions FC-B and 75EA-L-F10 inhibits MRSA biofilm production by preventing cell –surface attachment and reducing PBP2a level

Generally, the results of biofilm studies have shown that the selected bioactive fractions, FC-B and 75EA-L-F10 have anti-biofilm properties, as these fractions suppressed biofilm production. 75EA-L-F10 showed significant improvement in inhibiting biofilm production when combined with ampicillin. However, combination treatment of FC-B and ampicillin did not demonstrate a marked inhibition on biofilm production compared to treatment with FC-B alone. Hence, suggesting that FC-B may be solely responsible for the anti-biofilm properties observed. Elimination of virulent factor such as biofilm forming capacity may generate better antimicrobial activity as compared to activity of ampicillin alone which could have contributed improved suppression on biofilm production in combination treatment 75EA-L-F10 and ampicillin.

Study on cell-surface attachment indicated that these fractions are capable of preventing attachment of MRSA cell to the growth surface with the highest inhibition seen at MIC. At lower concentrations, the inhibition was approximately 50%. The ability of FC-B and 75EA-L-F10 to prevent cell-surface attachment is thought to reduce biofilm production. Attachment of the bacterial cells to its growth surface is the initial step in development of biofilm (O'Toole et al. 2000; Watnick and Kolter 2000) and this factor influences the final mass of biofilm production. A lesser cell-surface attachment at the initial stage reduces the number of bacteria involved in biofilm development subsequently decreases biofilm production or result in weak biofilm structure that may be easily eradicated (Overhage et al. 2008).

This is in agreement for results of biofilm studies in this chapter. From the correlation curves (see Figure 4.14) we were able to demonstrate that strong correlation

exists between cell-surface attachment and biofilm production for treatment of FC-B. Treatment with 75EA-L-F10 showed a weaker correlation for the same factors. This finding suggests that prevention of cell-surface attachment is one of the mechanisms involved in inhibition of biofilm production by the bioactive fractions in this study. In *S. aureus*, surface proteins such as MSCRAMMs and FnBPs induce cell motility and promote the bacterial cells to attach to a surface. The attachment process is also catalyzed by a group of enzyme called sortases (Schröder et al. 2006; Otto 2008). As such, prevention of cell-surface attachment by FC-B and 75EA-L-F10 could be related to the ability of these fractions to interact with proteins involved in the attachment process. Figure 4.16 describes the proposed mechanisms of the bioactive fractions in suppressing biofilm production in this study.

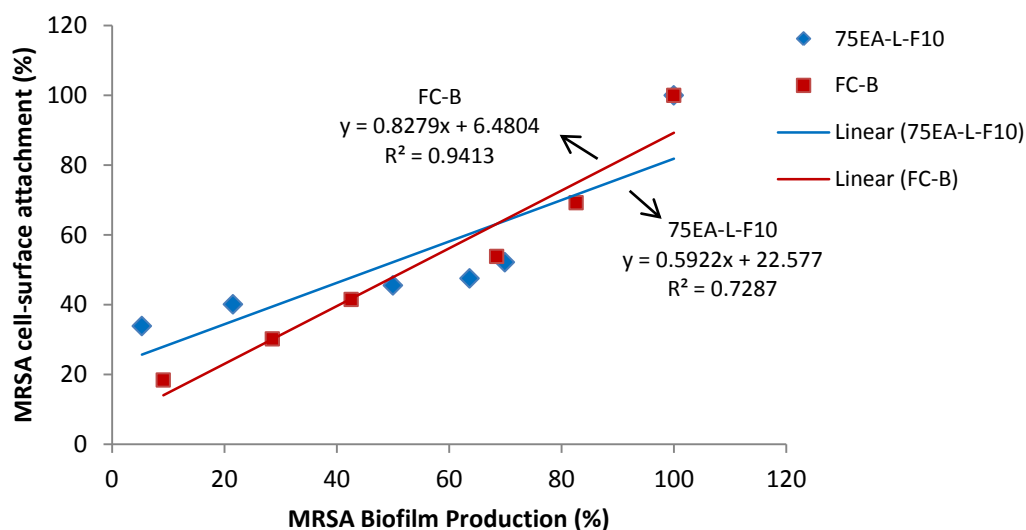


Figure 4.14 Correlation curves showing relationship between MRSA cell surface attachment and biofilm production for treatments of FC-B and 75EA-L-F10. R^2 value of each curve is depicted on the graph.

In determination of biofilm phenotype produced by MRSA strain used in this study, we found out that 77.36% of the biofilm dispersed when treated with proteinase K. The remaining 22.64% of biofilm matrix after the treatment are suspected to be other

essential structural composition of the biofilm such as DNA and polysaccharide (Costerton 2004; Rice et al. 2007; Otto 2008). The high dispersal of biofilm caused by proteinase K in this experiment indicates proteinaceous nature of MRSA biofilm. This is consistent with observations from previous studies that reported occurrence protein-based biofilm in MRSA since in these strains are often found to be dependent on the expression on FnFBs in contrast to PIA/PNAG in MSSA (Rice et al. 2007; Kaplan et al. 2012; Pozzi et al. 2012). The switch of the phenotype is caused by high level of PBP2a expression that represses *icaADBC* genes which are involved in PIA/PNAG-based biofilm production, and consecutively after a series of molecular events, promotes a PBP2a-mediated biofilm formation (Pozzi et al. 2012) (see Figure 4.15). Hence, we pursued to investigate if inhibition of PBP2a affects the biofilm production in our study.

Results of the semi-quantitative analysis of PBP2a latex agglutination test revealed that FC-B and 75EA-L-F10 reduced PBP2a level in MRSA biofilms. Subsequently, the biofilm production in presence of these fractions both alone and in combination with ampicillin was shown lower (ranging from 29.01 to 45.55%) compared to treatment with ampicillin alone. Earlier in Chapter 3, we have demonstrated that both of these fractions attenuated the level of PBP2a in MRSA based on Western blot experiments. It was previously suggested that PBP2a mediates biofilm production in MRSA, while the altered cell wall structure of MRSA that expresses PBP2a promotes cell-cell interactions (Pozzi et al. 2012). Although the mechanism by which PBP2a promotes MRSA biofilm production remains unclear it is known that cell-cell interaction is an important step in multilayer structure assembly in the development of biofilm (O'Toole et al. 2000; Pozzi et al 2012). Therefore, we propose that reduction of PBP2a level by FC-B and 75EA-L-F10 adversely affected cell-cell interaction and this leads to a disruption in biofilm production (see Figure 4.16).

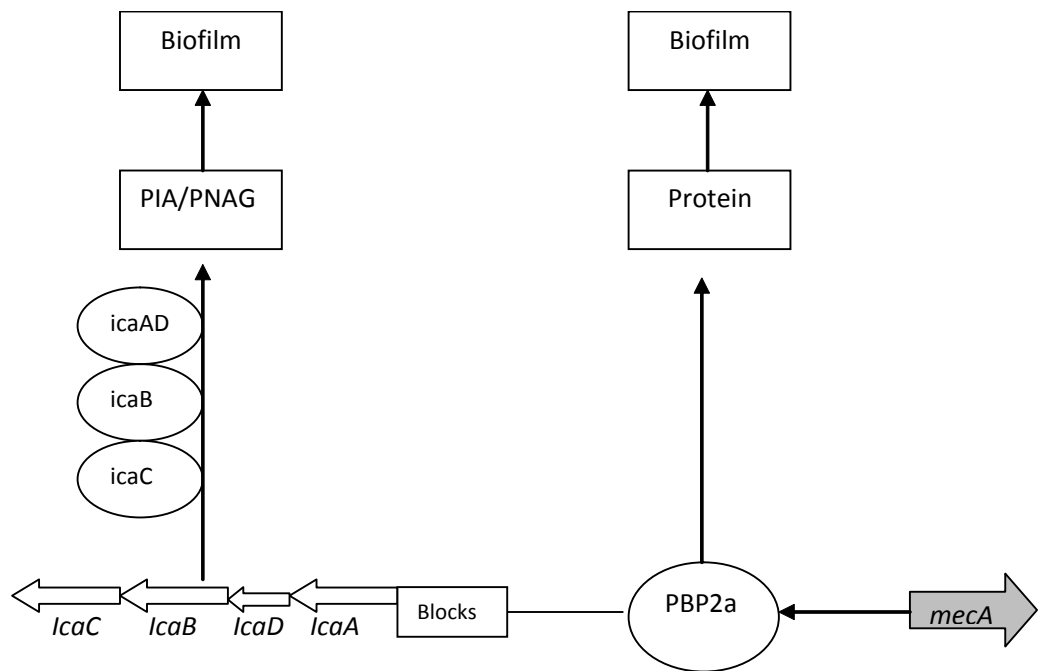


Figure 4.15 Model of PBP2a-mediated biofilm expression in MRSA. Presence of high level of PBP2a in MRSA represses the *icaADBC* blocking PIA/PNAG production which in turn correlates a PBP2a promoted biofilm formation.

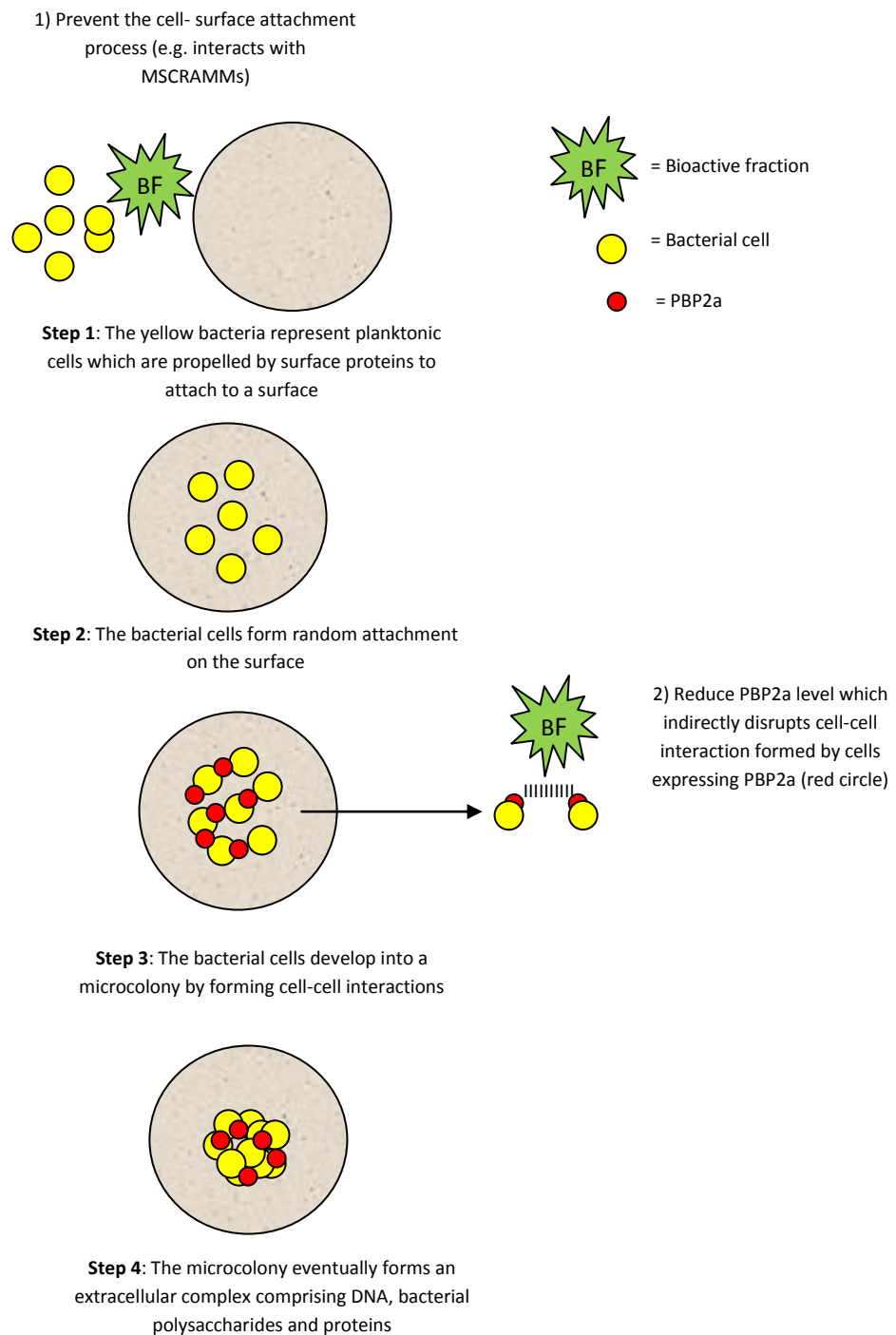


Figure 4.16

Schematic diagram showing steps involved in biofilm formation. The inhibition of biofilm production by the bioactive fractions (BF) is proposed due to 1) prevention of cell-surface attachment and 2) reduction of PBP2a level based on the results of this study.

4.5.2 MIC ampicillin inhibits MRSA biofilm production

MIC of ampicillin which was included as control, effectively inhibited MRSA biofilm production as high as 84.49% however, microtiter attachment assay revealed 62.20% of cell-surface attachment in the same treatment. Findings from microtiter attachment assay clearly indicated the inferior activity of ampicillin in preventing cell-surface attachment which occurs in first hour of incubation. This suggest that ampicillin does not act rapidly in obstructing the cell-surface attachment unlike the bioactive fractions thus, indicating a possible delayed antibacterial action by ampicillin. Based on this inference, we hypothesized that due to the delayed antimicrobial action; MRSA cells were observed to attach to the surface initially. However, continuous exposure to MIC of ampicillin (up to 24 hours in inhibition of biofilm production assay) may inhibit or slows the bacterial growth which subsequently reduced the final production of biofilm. Nonetheless, this does not suggest that ampicillin is a good anti-biofilm agent because delay in antibacterial action leads to prolonged and repeated exposure of MRSA to the antibiotic which is likely to contribute to the emergence of multi-drug resistance (O'Toole et al. 2000; Mataraci and Dosler 2012). Furthermore, the MIC of ampicillin used to treat the MRSA cultures was 50 µg/ml which exceeded MIC breakpoints for susceptibility to beta-lactam drugs (Brown 2001). Therefore, ampicillin is not indicated for treatment of biofilm related infections.

We also observed a sudden increase (4-fold) in MRSA biofilm production when the concentration of ampicillin was reduced from MIC to $1/2 \times \text{MIC}$. As described in earlier findings, sub-MIC of beta-lactams antibiotics in fact promotes formation of biofilm in *S. aureus in vitro* as much as 4-fold which we found consistent with results of our study. The amplification of biofilm production at sub-MICs of cell-wall active antibiotics such as ampicillin, was reported common and the reason was suggested due

to global cell stress that leads to physiological changes. In response to these changes, the bacteria act to protect the cells by forming biofilms (Carsenti-Etesse et al. 1992; Mah and O'Toole 2001).

4.5.3 High PBP2a level in biofilm from MRSA cultures treated with MIC ampicillin did not promote biofilm production

Attenuation of PBP2a level in MRSA biofilm appeared to reduce biofilm production for treatments with the bioactive fractions. Likewise, higher level of PBP2a in biofilm treated with ampicillin alone (at $1/32 \times \text{MIC}$ and $1/64 \times \text{MIC}$) corresponded to increased biofilm production (Table 4.2). However, the same was not observed for MIC of ampicillin. Despite the occurrence of high level of PBP2a in the biofilm extract, MIC of ampicillin was observed to markedly reduce biofilm production. The reason for this observation is because the MIC of ampicillin supposedly slowed the bacterial growth that consequently led to low biofilm formation. Furthermore, higher concentration of ampicillin may have effectively suppressed PBPs activity (other than PBP2a) that is required for biofilm production. Recent research reported that PBPs are involved in biofilm formation and motility of bacterial cells (Kumar et al. 2012; Ouyang et al. 2012). Due to these reasons, we presume that the high expression of PBP2a in biofilm treated with MIC of ampicillin was unable to promote biofilm formation. Even though MIC of ampicillin was shown to inhibit biofilm production, the aggravation of PBP2a level at this concentration indicates increased resistance that may induce more complications in treatment of biofilm in the long run.

4.5.4 Conclusion

Overall we have demonstrated that FC-B and 75EA-L-F10 inhibited biofilm formation in MRSA by preventing cell-surface attachment and interrupting PBP2a expression, which indirectly disrupts cell-cell interaction that is also necessary for biofilm development. In combination treatments, a significant inhibition of biofilm formation was only observed for combination of ampicillin and 75EA-L-F10. Nevertheless, both 75EA-L-F10 and FC-B was found to possess anti-biofilm properties which may have contributed to improved antimicrobial activity as the inhibition of biofilm is expected to result in increased uptake of the antibiotic among the biofilm protected cells. Besides affecting the two studied factors in this chapter, FC-B and 75EA-L-F1 may also affect other factors involved in MRSA biofilm formation such as role of wall teichoic acids, quorum sensing process and global stress response (Mah and O'Toole 2001; Overhage et al. 2008; Holland et al. 2011).

Analysis of biofilm production and cell-surface attachment showed a positive correlation for treatments with the bioactive fractions alone and in combination with ampicillin. Hence, suggesting that prevention of cell-surface attachment is a useful strategy in combating biofilm formation. Whereas reduction of PBP2a level in the biofilm affirmed our previous finding (Chapter 3) that these bioactive fractions attenuated PBP2a. The current finding indicated that PBP2a plays a crucial role in mediating the formation of biofilm which renders further resistance in these sessile biofilm populations of MRSA. As such, the ability of FC-B and 75EA-L-F10 in attenuating PBP2a is valuable characteristic for development of new treatment for MRSA.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

5.1 BACKGROUND

Resistance issue of MRSA continues to threaten the world population despite the availability of new antibiotics (David and Daum 2010). Production of PBP2a accompanied by biofilm-forming capacity contributes to the prevalence of MRSA infections. Beta-lactams that block PBPs action in assembly and regulation cell wall biosynthesis are no longer effective. This is because PBP2a which has a lower binding affinity to these beta-lactams overtakes the blocked PBPs functions and ensures a smooth cell wall synthesis in MRSA (Berger-Bächi 1994; Berger-Bächi and Rohrer 2002). The biofilm forming capacity, on the other hand, plays a fundamental role in protecting bacterial cells from potential antimicrobial agents in the sessile stage. Besides, bacterial biofilm contributes to the manifestation of antibiotic resistances (Costerton et al. 1999; Petrelli et al. 2006; Del Pozo and Patel 2007) which further increases the challenges in treating MRSA infections.

Considering MRSA's resistance, future quest of antimicrobials should be focused in finding solutions to overcome these virulent factors. In pharmacology, multi-drug therapy which is dubbed as synergism strategy has effectively curb resistance issues specifically for treatment of infectious diseases such as tuberculosis (Wagner and Ulrich-Merzenich, 2009). Natural products from plants are promising candidates for synergistic treatment as these compounds demonstrated ability to modify resistance displayed by microorganism such as production of enzymes, MDR efflux pump system and altered target site (Hemaiswarya et al. 2008). Restoration of antimicrobial efficacy of conventional drugs against the antibiotic resistant pathogens can be achieved when these plant products are used in combination.

In our earlier study, we have shown that ethyl acetate extract from *A. wilkesiana* and *D. grandiflora* possessed anti-staphylococcal activity (Othman et al. 2011a; Othman et al. 2011b). Following that, the current research was designed to evaluate effects of these plant extracts and their bioactive fractions in combination with ampicillin on MRSA. The results revealed that extracts and fractions from both plants demonstrated synergism with ampicillin in suppressing MRSA growth. Mode of action study showed that the selected bioactive fractions, FC-B and 75EA-L-F10, were able to attenuate PBP2a expression. In biofilm studies, these bioactive fractions inhibited MRSA biofilm production via prevention of cell-surface attachment and reduction of PBP2a level which may have indirectly disrupt the cell-cell interaction in biofilm development. HPLC analysis revealed that these bioactive fractions are complex mixtures of plant metabolites. While phytochemical analysis, showed presence of biologically active phytochemicals such as tannins, saponins, alkaloids, flavonoids, sterols/steroids, and glycosides.

5.2 SYNERGISM OF CRUDE EXTRACTS *A. WILKESIANA* AND *D. GRANDIFLORA* WITH AMPICILLIN IN INHIBITING MRSA GROWTH

As shown in Chapter 3 crude extracts of *A. wilkesiana* and *D. grandiflora* (9EA and 75EA-L) reduced the MIC of ampicillin against MRSA from 50 µg/ml to 0.78 µg/ml and 1.56 µg/ml respectively. The kinetic growth curves illustrated a marked suppression on MRSA growth in combination treatments compared to treatment with one agent alone. From the FIC index interpretation, the combinations of these crude extracts and ampicillin indicated synergism interaction based on FIC indices < 0.5.

The combinatorial activities of plant extracts or products with antibiotics in which synergism was present is principally attributed to resistance modifying action of

the plant test agents (Abreu et al. 2012). The resistance modifying action of plant products are implied to attenuate the bacterial resistance mechanism, and hence promote the antimicrobial action of antibiotics in combination treatments (Shahverdi et al. 2007). This is supported by the findings that plant produces secondary metabolites in response to microbial attack in nature where some of these products were shown to fight infections successfully (Sibanda and Okoh 2007). In agreement, phytochemicals analysis revealed presence of tannins, saponins, alkaloids, flavonoids, sterols/steroids, and glycosides in 9EA and 75EA-L. These are pharmacologically active phytochemicals that were demonstrated to influence bacterial resistance mechanisms *in vitro* (Abreu et al. 2012).

The phytochemicals occurring in 9EA and 75EA-L are suspected to give rise to the observed synergism in the combination treatments. Since these extracts contains mixture of compounds, it is highly possible for these extracts to have multi targets such as bacterial cell membranes or wall, metabolic pathways, production of protein, and DNA replication in exhibiting antimicrobial action. Therefore, the mechanism underpinning antibacterial and synergistic effects of 9EA and 75EA-L is unclear at this stage of experiment. However, previous studies reported that plant metabolites classified as tannins, flavonoids and alkaloids are capable of inhibiting MRSA or *S. aureus* growth via synergism with antibiotics and some were found to attenuate the resistance mechanisms (Table 5.1). Besides that, other phytochemicals found in the extracts namely sterols and glycosides were reported to demonstrate antimicrobial activities against MRSA (Nazemiyeh et al. 2008; Singh et al. 2012).

Table 5.1 Synergistic activities of selected phytochemicals and their mode of actions against MRSA and *S. aureus*

Phytochemical	Antibacterial activity	Mode of action	Reference
Tannins	Act synergistically with oxacillin	Prevent PBP2a synthesis and secretion of beta-lactamase in MRSA	Yam et al. 1998 Rocarro et al. 2004 Shibata et al. 2005 Marquez et al. 2005 Hu et al. 2002
Alkaloids	Reduce MIC of norfloxacin in combination treatments	Inhibit MDR efflux pump mechanism in <i>S. aureus</i>	Markham et al. 1999 Gibbons et al. 2003 Stavri et al. 2007
	Produced an additive and synergistic effects when combined with ampicillin and oxacillin	Increase membrane permeability and intercalate into DNA	Yu et al. 2005 Lewis and Ausubel 2006
Flavonoids	Reduced MIC oxacillin against MRSA in combination treatments	Target MRSA cell membrane and cell wall	Hatano et al. 2005

The synergism observed in combination treatments pinpoints that these phytochemicals could affect the resistance mechanisms in MRSA. This phenomenon is thought to improve antibacterial potency of ampicillin. Therefore, a lower MIC of ampicillin was recorded in combination treatments. Following that, the fractions obtained from 9EA and 75EA-L were evaluated for synergistic activity. Effects of combination treatment on a MRSA resistance determinant; PBP2a was carried out for selected bioactive fractions. The selected bioactive fractions were also evaluated for inhibitory effect on MRSA biofilm since PBP2a (encoded by *mecA* gene) influences biofilm phenotype indicating interrelatedness of these virulent factors (McCarthy et al. 2015).

5.3 EFFECTS OF BIOACTIVE FRACTIONS ON PBP2a

5.3.1 FC-B

Fraction FC-B is a fraction obtained from 9EA, crude extract of *A. wilkesiana*. The fraction has a MIC of 3 mg/ml against MRSA and presence of this fraction ($1/4 \times \text{MIC} = 0.75$ mg/ml) enhanced the potency of ampicillin up to 32-fold by reducing MIC ampicillin from 50 $\mu\text{g/ml}$ to 1.56 $\mu\text{g/ml}$ for MRSA. Synergy was indicated present by FIC index interpretation in which the scored index was 0.28 ($< 0.5 = \text{synergism}$) for combination of $1/32 \times \text{MIC}$ ampicillin + $1/4 \times \text{MIC}$ FC-B. The growth curve experiments likewise revealed an increased antimicrobial action when FC-B was combined with ampicillin. From the Western blot experiments (3), the synergistic activity observed between FC-B and ampicillin against MRSA was shown to be associated with inhibition of PBP2a. This suggests that FC-B can either inhibit the production of PBP2a or directly inactivate PBP2a.

Studies on *A. wilkesiana* indicated that compounds from class of tannins were commonly found in extracts or fractions of this plant which demonstrated antimicrobial activity (Oladunmoye 2006; Gotep et al. 2010; Din et al. 2013a; Din et al. 2013b). Some of the well-known tannins such as corilagin and tellimagrandin I exceptionally reduced MICs of beta-lactams for MRSA when used in combination. The mode of action of these compounds was suggested through inactivation of PBP2a (Shiota et al. 2004). We postulated that corilagin is present in FC-B, since corilagin was previously isolated and identified by our colleagues from the same source of plant materials (Din et al. 2013b). This is further supported by phytochemical testing that showed the presence of tannins in FC-B. Based on the Western blot results, FC-B was confirmed to inhibit PBP2a

production in MRSA (either alone or in combination with ampicillin) which we found similar to mechanism of action of corilagin.

Tannins appeared to play an important role in antibacterial activity of FC-B. Along with corilagin, the researchers in our laboratory also identified other tannins; geraniin and ellagitannin in the *A. wilkesiana* fractions. Ellagitannin demonstrated synergistic activity with ampicillin against MSSA and the mechanism of action was proposed through bacterial cell lysis in which the bacterial cell wall was observed damaged (Din et al. 2013a). This shows that tannins may have cell wall or membranes as targets. In line with this, a previous study showed that epicatechin gallate (ECg- a tannin) which reversed beta-lactam resistance in MRSA did not interact through direct binding with PBP2a or influence its enzymatic activity. Instead ECg affects the fluid dynamics of the cytoplasmic membrane by decreasing the fluidity of the lipid bilayer. These changes trigger expression of genes involved in preservation and reparation of a compromised cell wall which eventually delocalized PBP2a from the cell wall biosynthesis process (Bernal et al. 2010).

At present, our studies indicate occurrence of tannins in FC-B could have largely contributed to attenuation of PBP2a. Further studies are necessary to determine the tannic compounds in FC-B and to elucidate how FC-B inhibits PBP2a production in MRSA. The current results leads to suggestions that active compounds from FC-B may 1) interact with MecR2 (anti-repressor) or MecR1 (sensor inducer) involved in expression of *mecA* gene encoding for synthesis of PBP2a in MRSA or 2) interact directly with PBP2a and cripples its role in cell wall of biosynthesis.

5.3.2 75EA-L-F10

Fraction 75EA-L-F10 is a major fraction isolated from the 75EA-L, crude extract of *D. grandiflora* leaves. MIC of 75EA-L-F10 versus MRSA is 0.75 mg/ml and combination of this fraction ($1/4 \times \text{MIC } 75\text{EA-L-F10} = 0.19 \text{ mg/ml}$) with ampicillin decreased MIC ampicillin by 64-fold (from 50 $\mu\text{g/ml}$ to 0.78 $\mu\text{g/ml}$). FIC index of 0.25 ($< 0.5 = \text{synergism}$) was scored in combination treatment of $1/64 \times \text{MIC ampicillin} + 1/4 \times \text{MIC } 75\text{EA-L-F10}$ suggesting synergism present between these two components. Similarly, the growth curve assay indicated that a better inhibition on MRSA growth was achieved in combination treatments in contrast to treatment with ampicillin or 75EA-L-F10 alone. Investigation of mode action (3) implied that the restoration of ampicillin's antimicrobial potency is related to ability of 75EA-L-F10 to inhibit PBP2a production which was identical to antimicrobial action of FC-B.

In contrast to *A. wilkesiana*, *D. grandiflora* is not widely studied for its antibacterial properties. The available literatures on this plant are mostly related anti ageing, skin whitening and anti cancer properties of this plant's extracts and compounds derived from it (Bhakuni et al. 1971; Sharma et al. 1972; Sharma et al. 1974; Tsukiyama et al. 2010; Kaweetripob et al. 2012). Hence, we could not specifically identify a group of phytochemical that is most likely to be responsible for PBP2a inhibition. Nevertheless, occurrence of tannins in 75EA-L-F10 explains the antimicrobial properties of this fraction to a certain extent, since tannin compounds have been extensively reported to exhibit anti-MRSA activity (see section 5.3.1). The mechanisms involved in inhibition of PBP2a by 75EA-L-F10 are elusive, but similar to FC-B we postulate that 75EA-L-F10 could affect the expression of *mecA* gene or degrade the resistant protein.

Interestingly, flavonoids that was absent in FC-B, was found present in 75EA-L-F10. Similar to tannins, several flavonoids such as licoricidin, licochalcone, glyasperins, and glabridin demonstrated synergism with oxacillin in halting MRSA growth (Hatano et al. 2005). Study of mechanism of action revealed that these compounds were not affecting PBP2a production. Instead they were suggested to interact with MRSA cell membrane or wall which gave rise to the observed antimicrobial and synergistic (with oxacillin) activity (Hatano et al. 2005). This implies that flavonoids in 75EA-L-F10 could be one of the potential phytochemicals which contributed to the antimicrobial and synergistic activity of the fraction.

5.4. EFFECTS OF BIOACTIVE FRACTIONS ON BIOFILM

The resistance to beta-lactam antibiotics particularly expression of PBP2a seemed to be crucial in determination of the biofilm phenotype (McCarthy et al. 2015). Hence, the bioactive fraction FC-B and 75EA-L-F10 were evaluated for anti-biofilm activities. Our studies showed that these fractions inhibited biofilm formation via 1) prevention of cell-surface attachment and 2) reduction of PBP2a level that is necessary for cell-cell interaction in development of biofilm. Additionally, we demonstrated that biofilm produced by MRSA strain used in this study is proteinaceous.

The biofilm inhibiting properties of FC-B and 75EA-L-F10 are attributed to presence of the phytochemicals. Tannins are phytochemicals commonly found in all plants, exert antibacterial effects by interacting with bacterial cell wall or membrane. They are generally able to penetrate biological membranes easily therefore prevent clustering of bacterial cells which negatively affects biofilm production (Blanco et al. 2005; Cushnie and Lamb 2011). Besides, compounds classified as tannin such as epigallocatechin gallate (EGCg) is capable of binding to peptidoglycan and destroy the

bacterial cell wall integrity. The weakened cell integrity may hinder the initial phase of biofilm production that is the interaction between the bacterial cell wall and the surface for attachment (Carpentier and Cerf 1993; Zhao et al. 2001; Zhao et al. 2002; Yoda et al. 2004). A recent study that showed tannic acids influence *S. aureus* cell surface hydrophobicity which is an important factor in cell-surface attachment in the process of biofilm formation (Chusri et al. 2012). There is also evidence that tannins interfere with bacterial quorum-sensing system that catalyze the biofilm formation (Huber et al. 2003). It is therefore highly plausible that the occurrence of tannins in FC-B and 75EA-L-F10 were responsible for the observation made in the microtiter attachment assay, in which the fraction remarkably reduced MRSA cell-surface attachment.

Other than tannins, flavonoids which were found only in 75EA-L-F10 are suspected to play a vital role in the anti-biofilm activities observed. Previously, flavonoids were reported to target cell membrane which subsequently weakens the integrity of biofilm structure. It was shown that flavonoid components in propolis extracts affect bacterial membrane potential (changes in the permeability) and inhibit bacterial motility (Mirzoeva et al. 1997). Inhibition of bacterial motility especially reduces the ability of bacterial cells to move towards a surface for the attachment process. This eventually results in lower cell-surface attachment as what we have observed in microtiter attachment assay. Besides, influence of flavonoids on membrane permeability may have led to increased antibiotic uptake into the biofilm protected cells and ultimately result in growth inhibition. In a different study, flavonoids were reported to affect the sortase activity, which is known to influence the adhesive property of bacterial cell wall, leading to interruption of biofilm development (Tiwari and Sen 2006; Mataraci and Dosler 2012). Hence, events that are described here could be one of the potential mechanisms which contributed to the anti-biofilm activities in 75EA-L-F10.

Besides affecting the cell-surface attachment process, phytochemicals occurring in FC-B and 75EA-L-F10 were found to inhibit PBP2a production (section 5.3). The attenuation of PBP2a by these fractions influenced the level of PBP2a in MRSA biofilm (PBP2a latex agglutination assay). Since PBP2a was earlier hypothesized to promote biofilm production in MRSA (Pozzi et al. 2012), reduction of PBP2a level in biofilm by FC-B and 75EA-L-F10 is thought to obstruct cell-cell interaction involved in biofilm development.

We also found that in biofilm studies, combination of FC-B and ampicillin did not enhance the anti-biofilm activity considerably compared to activity of the fraction alone. Combination of 75EA-L-F10 and ampicillin however resulted in a significant decrease in biofilm production. Nevertheless, the ability of both fractions in inhibiting biofilm production implies elimination of virulent factor which overall may have result in improved antibacterial effects.

5.5 ANTIMICROBIAL ACTION OTHER BIOACTIVE FRACTIONS

Along with FC-B and 75EA-L-F10, other bioactive fractions 9EA-FB, 9EA-FD and 75EA-B-F4 were identified in this study. Nevertheless, due to problems such as low yield and unstable activity as described in Chapter 3, some of these fractions and their derivatives were eliminated from the experiments. Furthermore, since the aim of this study is to identify bioactive fractions which have synergistic action with ampicillin, fractions that did not display the desired activity or have weaker synergism were excluded from the study. Fraction 9EA-FB and 75EA-B-F4 both inhibited MRSA growth but did not demonstrate any synergism with ampicillin. Whereas, 9EA-FD initially had moderate anti-MRSA activity however the sub-fractions obtained after VLC/CTLC were inactive in the biological assays and some were inadequate in amount.

5.6 CONCLUSION AND FUTURE STUDIES

5.6.1 Summary

Findings in this study showed that *A. wilkesiana* and *D. grandiflora* yields bioactive fractions that have antibacterial effects on MRSA and MSSA. The antibacterial potential of the crude extracts and the fractions explains the effectiveness of the plant extracts used traditionally to heal infectious diseases. Combining results from HPLC and phytochemical analysis together with information from literature reviews, we deduced that the antibacterial activity was observed as a result from presence of phytochemicals mainly tannins and flavonoids. These plant metabolites could target multiple factors that are essential for MRSA growth and survival (see Figure 5.1). On the other note, our study is the first to report the ability of fractions from *A. wilkesiana* and *D. grandiflora*; 1) to work synergistically with ampicillin in suppressing MRSA growth via PBP2a inhibition and 2) to inhibit MRSA biofilm production by preventing cell-surface attachment and reducing PBP2a level which is necessary for cell-cell interaction in biofilm development. The synergistic activities and the ability of the plant metabolites from *A. wilkesiana* and *D. grandiflora* in attenuating virulent factors in MRSA (PBP2a production and biofilm forming capacity) indicate the potential use these plants metabolites as adjuvant for antibiotics to reverse beta-lactam resistance in MRSA.

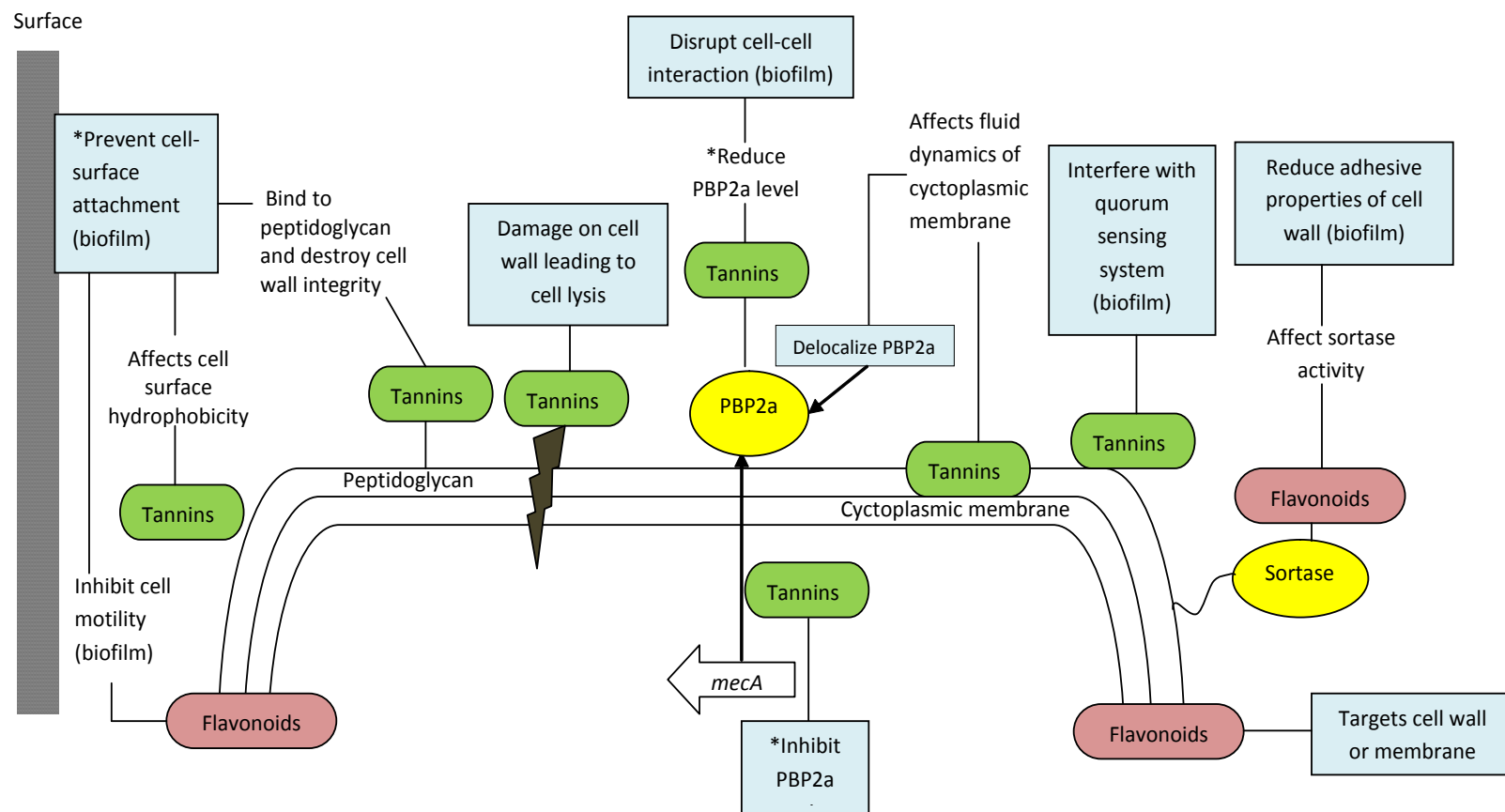


Figure 5.1

A schematic diagram representing multiple targets of tannins and flavonoids in inhibiting bacterial/MRSA growth and biofilm formation based on previous studies. Tannins were found in both fractions from *A. wilkesiana* (FC-B) and *D. grandiflora* fraction (75EA-L-F10). Flavonoids were only found in 75EA-L-F10. Mechanisms of anti-biofilm and antibacterial action demonstrated by the bioactive fractions FC-B and 75EA-L-F10 in this study are labeled as *.

5.6.2 Future studies

The current study has enabled finding of two bioactive fractions, FC-B and 75EA-L-F10. Nonetheless, no active compound in these fractions was identified. Corilagin, geraniin and ellagitannin were highly likely to occur in FC-B based on their discovery in *A. wilkesiana* extracts by another group in our laboratory. However, it is essential to identify the active compounds in these fractions for a conclusive study of mode of anti-MRSA action. Furthermore, in order to commercialize herbal extract or plant products, it is important to standardize the active ingredients to maintain the clinical efficacy of the product. In the mean time, no antimicrobial compound has been isolated from *D. grandiflora* extracts so far, hence this opens a new facet of study that potentially could lead to discovery of novel compounds.

Mechanism of action study revealed that components from FC-B and 75EA-L-F10 inhibit PBP2a production. We postulated that this could be due interruption in *mecA* gene expression that involves the anti-repressor protein MecR2 or the sensor-inducer domain MecR1. This requires further molecular studies involving experiments such as DNA manipulations, PCR, ELISA, and transcriptional analysis as conducted in a previous research (Arède et al. 2012). These experiments would be useful to study protein interactions and protein content. In addition, since in *S. aureus* species are known to produce at least four types of PBPs, effects of these fractions on other PBPs which can be studied via Western blot experiments may provide better understanding of mechanism of action. Another interesting dimension of study is the effects of the bioactive fractions on beta-lactamase which is an enzyme that confers resistance to penicillins in *S. aureus*. Apparently transcriptional control of *mecA* encoding for PBP2a and *bla_Z* encoding for beta-lactamase is regulated by homologous repressors which was demonstrated to cross-talk. Recently, the *bla* regulators were found to stabilize *mecA* acquisition (Oliveira

and de Lencastre 2011) and in fact, the *bla* regulatory system promotes optimal expression of *mecA* by disrupting the efficient MecI-mediated repression on *mecA* (Arède et al. 2013). Therefore, study on effects of the bioactive fractions on beta-lactamase production may explain the link between the two resistant determinants (PBP2a and beta-lactamase) in MRSA.

We also found that the studied bioactive fractions possess anti-biofilm activities related to prevention of cell-surface attachment and reduction in PBP2a level in biofilm. The production of biofilm in MRSA is influenced by multiple factors such as bacterial motility, surface proteins, quorum sensing systems, and global stress response. Hence, a future studies encompassing investigations of genes involved in biofilm development through implementation of DNA microarray techniques as shown in an earlier research (Overhage et al. 2008) may aid deeper understanding of antimicrobial action of the bioactive fractions in relation to biofilm production.

The observed anti-MRSA activity in the bioactive fractions and extracts is attributed to presence of phytochemicals. In this study, tannins, flavonoids and alkaloids are theoretically the major components that gave rise to antimicrobial activity. Based on preceding works, compounds belonging to these groups were widely reported to target cell wall and membrane. Thus, study of MRSA cell membrane polarization accompanied by microscopy examination on cell wall upon treatment with these bioactive fractions could provide evidence of interaction of the phytochemical components with bacterial cell wall or membrane.

Overall, this research has demonstrated synergism between bioactive fractions from *A. wilkesiana* and *D. grandiflora* with ampicillin in attenuating a resistant determinant namely PBP2a. Likewise these fractions also possess the ability to

overcome a virulent factor, biofilm production in MRSA. However since the bioactive fractions contains mixture of naturally occurring compounds the mechanism of antimicrobial action is very likely to be more complex and involves several targets. Therefore the current proposed mode of action as yet incompletely defined mechanism. Nevertheless, the presence of pharmacologically active phytochemicals indicates that these bioactive fractions may be good candidates for development of new treatment for MRSA.

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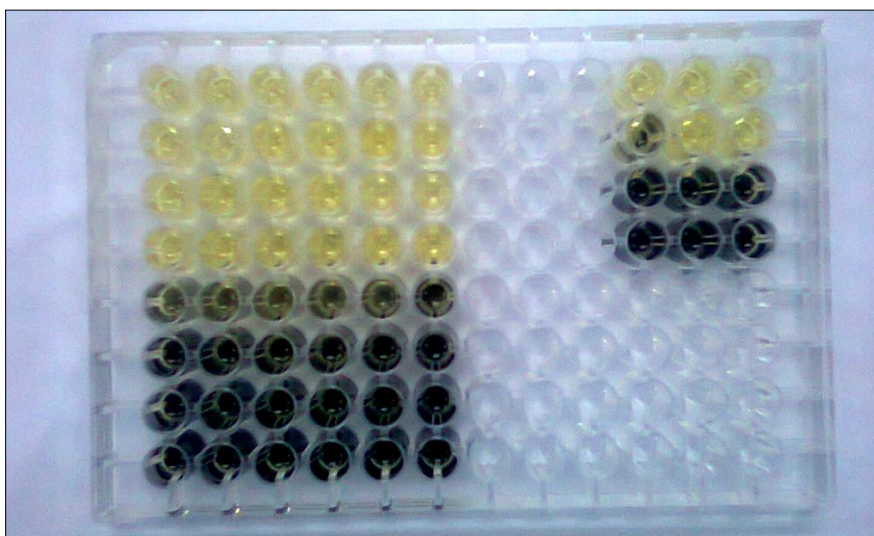
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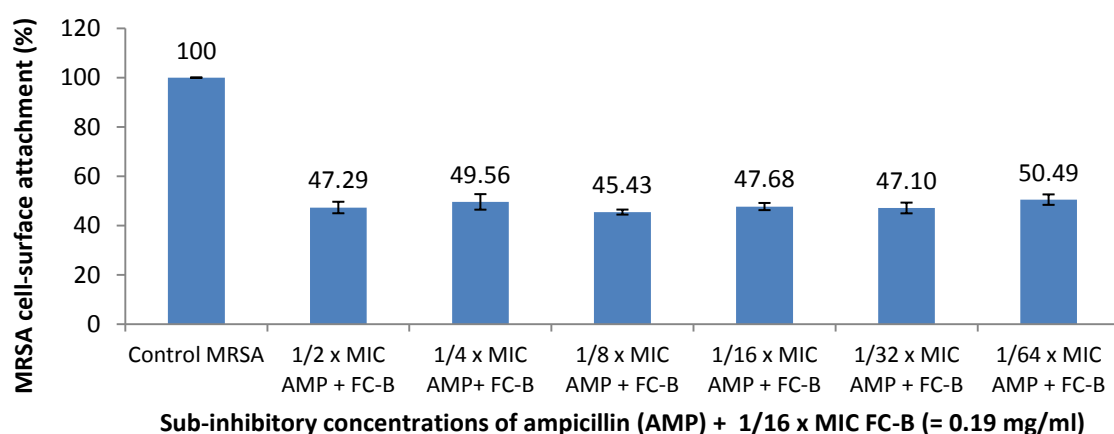
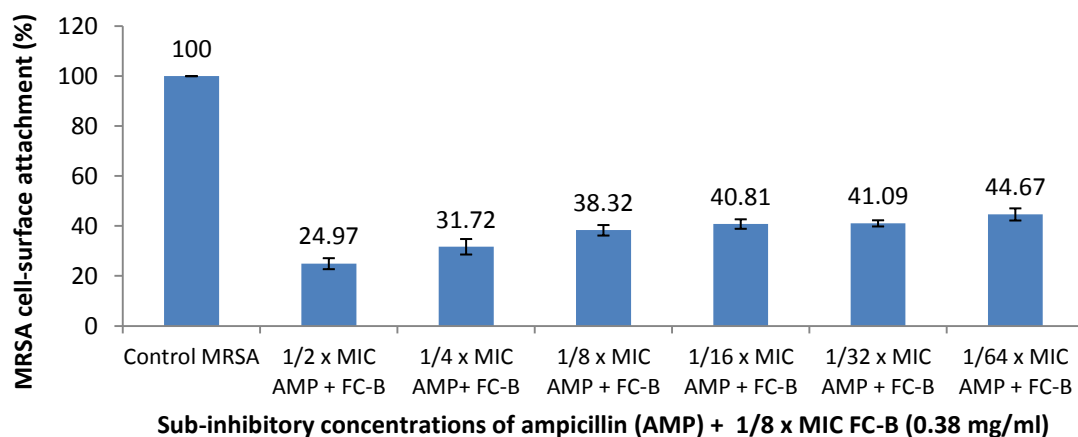
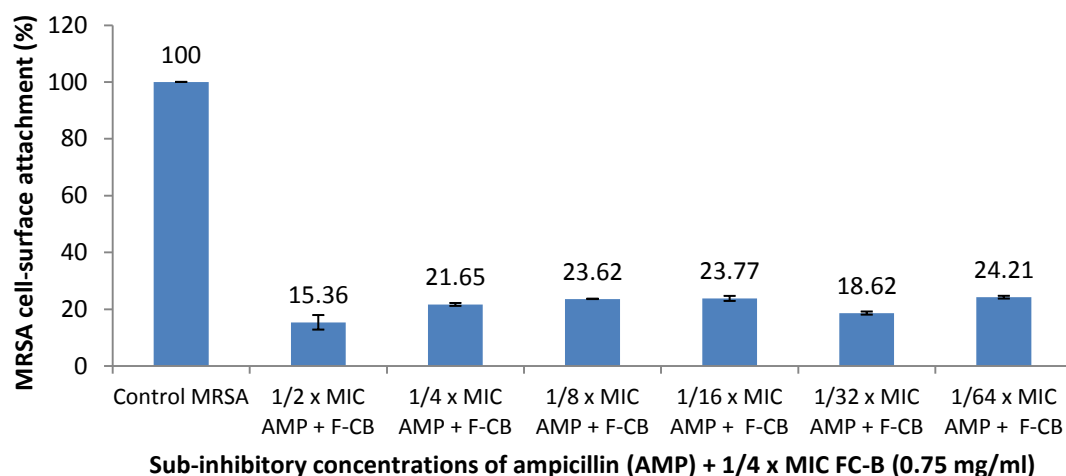
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APPENDICES

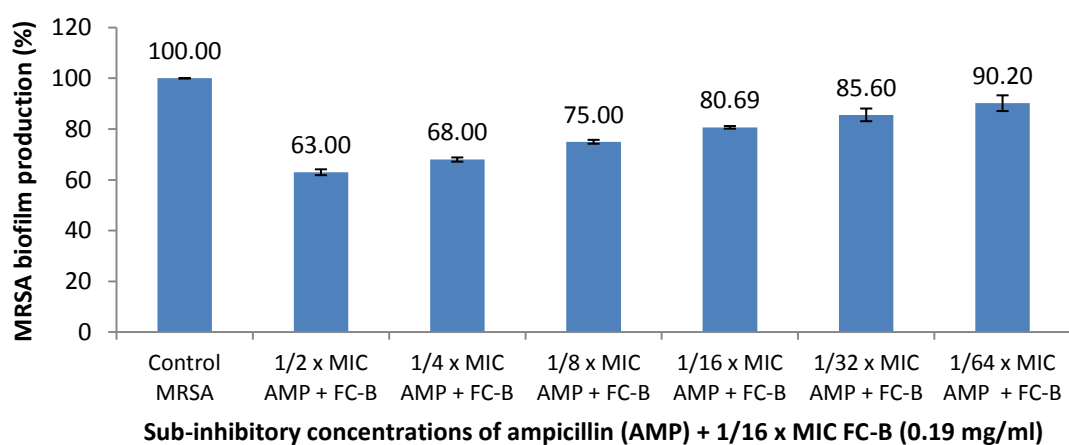
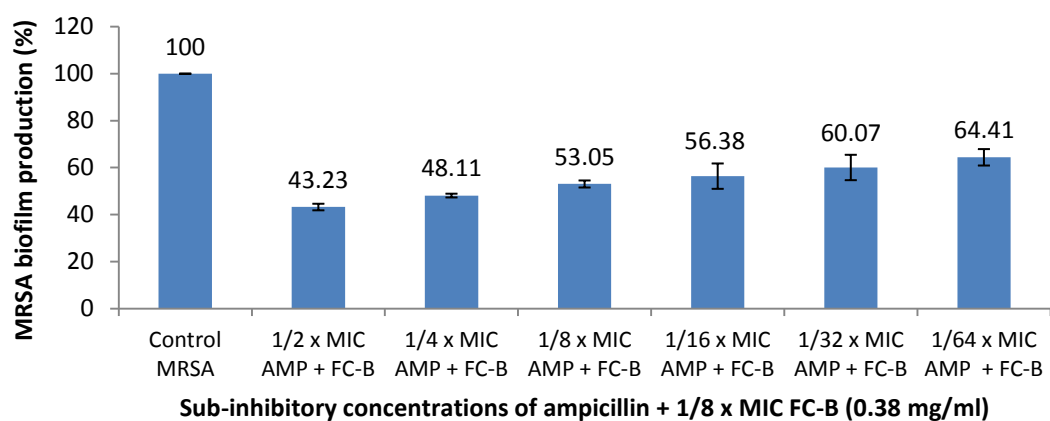
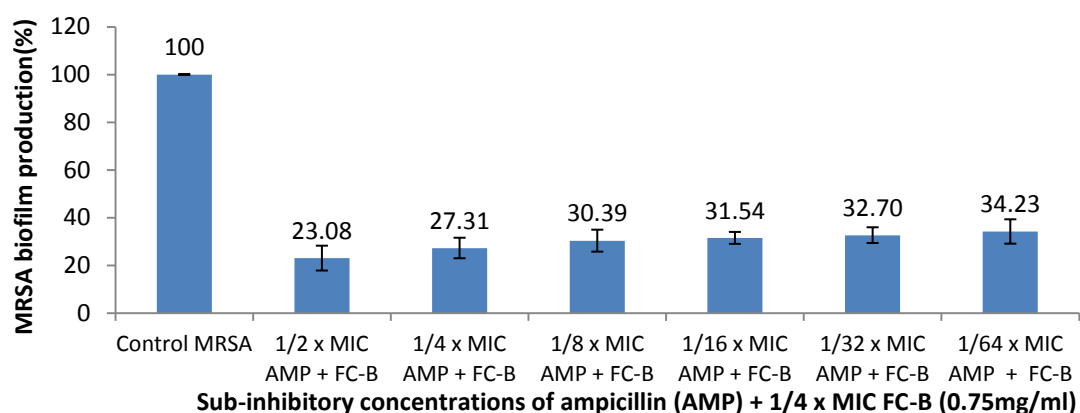
APPENDIX 1: Determination of MIC via broth microdilution assay, the lowest concentration of test sample in which no color changes was observed (sample in the well remain yellow after addition of MTT) is considered as the MIC.



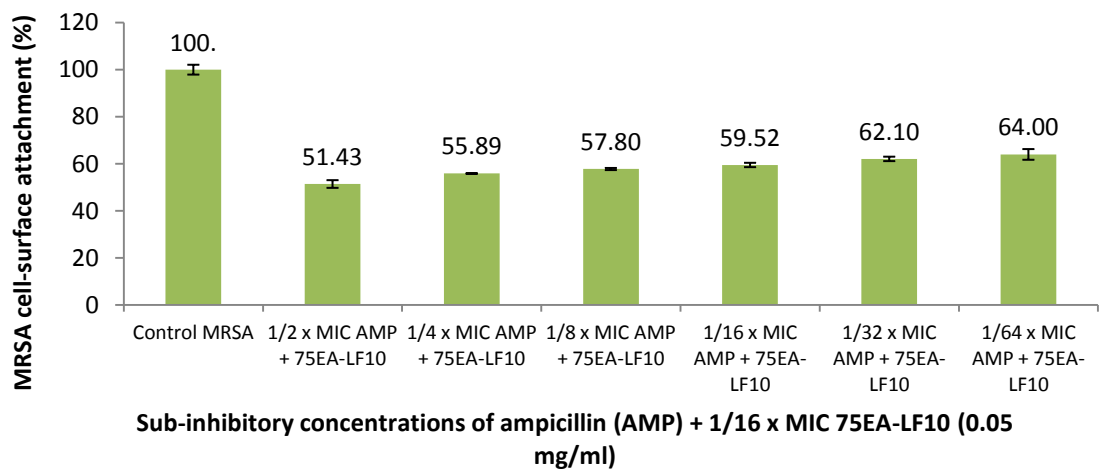
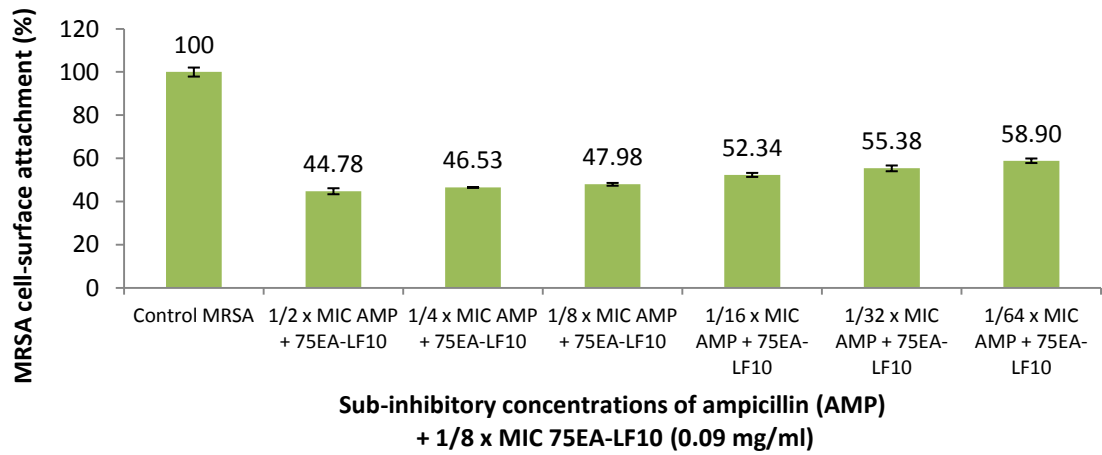
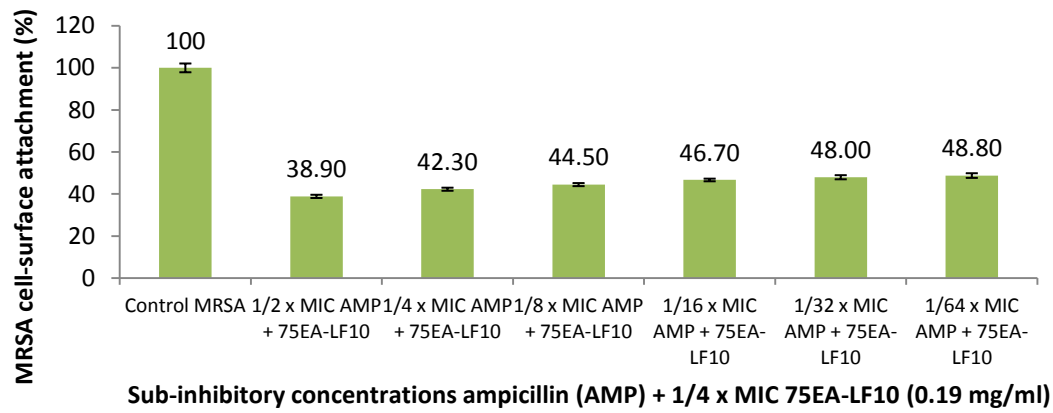
APPENDIX 2: MRSA cell-attachment (%) for combination treatments- ampicillin and FC-B



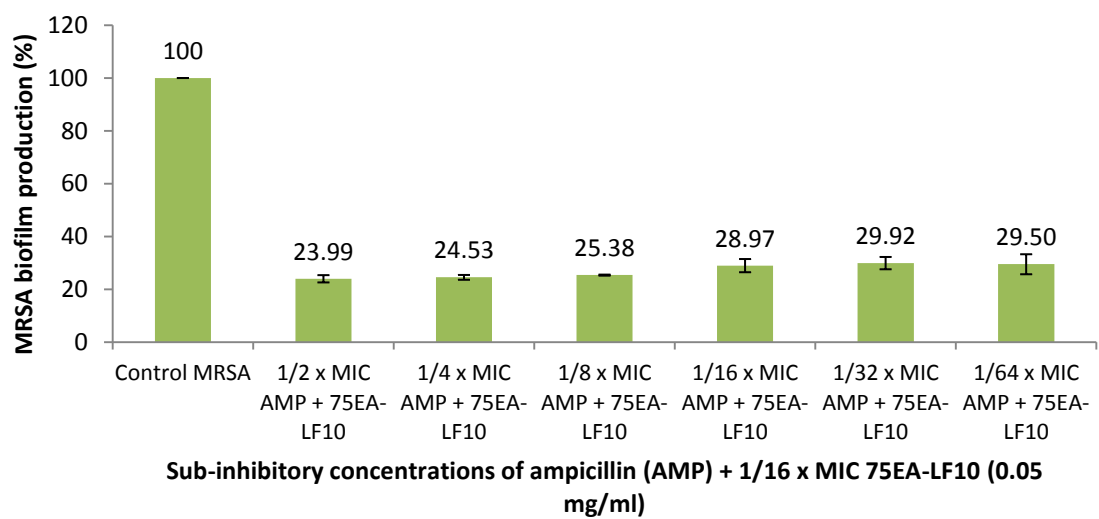
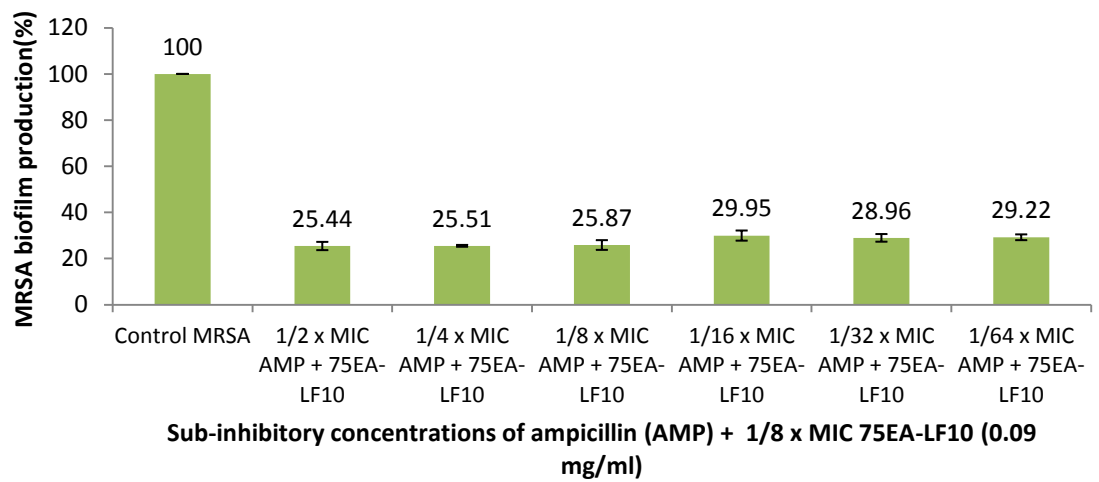
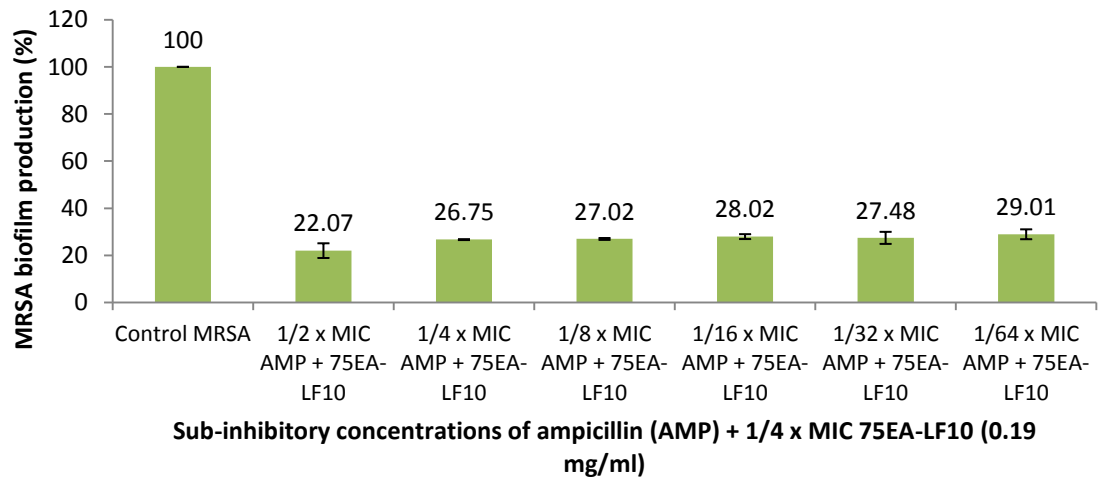
APPENDIX 3: MRSA biofilm production (%) for combination treatments- ampicillin and FC-B



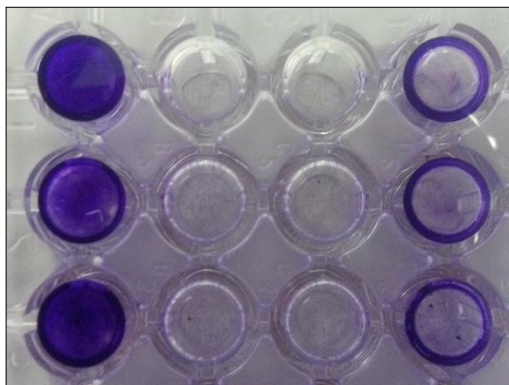
APPENDIX 4: MRSA cell-surface attachment for combination treatments (ampicillin and 75EA-L-F10)



APPENDIX 5: MRSA biofilm production (%) for combination treatments (ampicillin and 75EA-LF10)



APPENDIX 6: Crystal violet staining of microtiter plate for quantification of biofilm production



APPENDIX 7: TLC profile of fractions isolated from 9EA (*A. wilkesiana* crude extract) before being combined into major fractions based on their

